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(54) Title: FATTY ACYL-CoA: FATTY ALCOHOL O-ACYLTRANSFERASES

(57) Abstract

By this invention, a partially purified fatty acyl-CoA: fatty alcohol acyltransferase (wax synthase) is provided, wherein said protein is active in the formation of a wax ester from fatty alcohol and fatty acyl substrates. Of special interest is a jojoba embryo wax synthase having an apparent molecular mass of approximately 57kD. Also considered are amino acid and nucleic acid sequences obtainable from wax synthase proteins and the use of such sequences to provide transgenic host cells capable of producing wax esters.

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FATTY ACYL-CoA: FATTY ALCOHOL O-ACYLTRANSFERASES

This application is a continuation-in-part of USSN 07/796,256 filed November 20, 1991 and a continuation-in-part of USSN 07/933,411 filed August 21, 1992.

Technical Field

The present invention is directed to enzymes, methods to purify, and obtain such enzymes, amino acid and nucleic acid sequences related thereto, and methods of use for such compositions in genetic engineering applications.

INTRODUCTION

Background

Through the development of plant genetic engineering techniques, it is possible to transform and regenerate a variety of plant species to provide plants which have novel and desirable characteristics. One area of interest for such plant genetic engineering techniques is the production of valuable products in plant tissues. Such applications require the use of various DNA constructs and nucleic acid sequences for use in transformation events to generate plants which produce the desired product. For example, plant functional promoters are required for appropriate expression of gene sequences, such expression being either in the whole plant or in selected plant tissues. In addition, selective marker sequences are often used to identify the transformed plant material. Such plant promoters and selectable markers provide valuable tools which are useful in obtaining the novel plants.

A desirable goal which involves such genetic engineering techniques, is the ability to provide crop plants having a convenient source of wax esters. Wax esters are required in a variety of industrial applications, including pharmaceuticals, cosmetics, detergents, plastics, and lubricants. Such products, especially long chain wax esters have previously been available from the sperm whale, an endangered species, or

more recently, from the desert shrub, jojoba. Neither of these sources provides a convenient supply of wax esters. Thus, in order to obtain a reliable source of such compounds, transformation of crop plants, which are easily manipulated in terms of growth, harvest and extraction of products, is desirable.

In order to obtain such transformed plants, however, the genes responsible for the biosynthesis of the desired wax ester products must first be obtained. Wax ester production results from the action of at least two enzymatic activities, fatty acyl reductase and fatty acyl:fatty alcohol acyltransferase, or wax synthase. Preliminary studies with such enzymes and extensive analysis and purification of a fatty acyl reductase, indicate that these proteins are associated with membranes, however the enzyme responsible for the fatty acyl:fatty alcohol ligation reaction in wax biosynthesis has not been well characterized. Thus, further study and ultimately, purification of this enzyme is needed so that the gene sequences which encode the enzymatic activity may be obtained.

It is desirable, therefore, to devise a purification protocol whereby the wax synthase protein may be obtained and the amino acid sequence determined and/or antibodies specific for the wax synthase obtained. In this manner, library screening, polymerase chain reaction (PCR) or immunological techniques may be used to identify clones expressing a wax synthase protein. Clones obtained in this manner can be analyzed so that the nucleic acid sequences corresponding to wax synthase activity are identified. The wax synthase nucleic acid sequences may then be utilized in conjunction with fatty acyl reductase proteins, either native to the transgenic host cells or supplied by recombinant techniques, for production of wax esters in host cells.

Relevant Literature

Cell-free homogenates from developing jojoba embryos were reported to have acyl-CoA fatty alcohol acyl transferase activity. The activity was associated with a floating wax pad which formed upon differential centrifugation (Pollard *et al.* (1979) *supra*; Wu *et al.* (1981) *supra*).

Solubilization of a multienzyme complex from *Euglena gracilis* having fatty acyl-SCoA transacylase activity is reported by Wildner and Hallick (Abstract from *The Southwest Consortium Fifth Annual Meeting*, April 22-24, 1990, Las Cruces, NM.).

Ten-fold purification of jojoba acyl-CoA: alcohol transacylase protein is reported by Pushnik *et al.* (Abstract from *The Southwest Consortium Fourth Annual Meeting*, February 7, 1989, Riverside, Ca.).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The nucleic acid sequence and translated amino acid sequence of a jojoba fatty acyl reductase, as determined from the cDNA seqeunce, is provided in Figure 1.

Figure 2. The nucleic acid sequence and translated amino acid sequence of a jojoba wax synthase cDNA clone are provided.

SUMMARY OF THE INVENTION

By this invention, a partially purified fatty acyl-CoA: fatty alcohol *O*-acyltransferase protein, is provided, wherein said protein is active in the formation of wax esters from fatty alcohol and fatty acyl substrates. This fatty acyl-CoA: fatty alcohol *O*-acyltransferase is also referred to herein as "wax synthase". The wax synthase of this invention may be active with a variety of fatty acyl and fatty alcohol substrates, including acyl-CoAs and acyl-ACPs. The carbon chain length of these substrates may vary, although a given wax synthase may show preference for acyl and alcohol substrates having a specific chain length

or may be active with acyl and alcohol substrates having a wide range with respect to carbon chain length.

In general, the wax synthase of this invention has activity towards at least those acyl and alcohol substrates having a chain length of from 12 to 24 carbons, which carbon chain length may be represented by the formula "C_{2x}", where "x" is a number from 6 to 12, although other acyl or alcohol substrates may be tested and further activities discovered. In addition, having obtained the wax synthase protein of this invention, further manipulations are now possible as described in further detail below. These manipulations may lead to production or discovery of other related wax synthases.

Thus, in a first aspect, this invention relates to protein preparations demonstrating wax synthase enzymatic activity, and is exemplified by a seed-plant protein preparation. Such a preparation is produced by fractionation of jojoba embryos to produce a microsomal membrane preparation, solubilization of the wax synthase protein from this membrane preparation and further purification by chromatographic procedures. The jojoba wax synthase is shown herein to accept a broad range of acyl and alcohol substrates, which may be saturated or unsaturated (containing one or more double bonds between carbons). The activity of the jojoba wax synthase enzyme is given as E.C.2.3.1.75 in Enzyme Nomenclature 1984, with the recommended name "long-chain alcohol fatty-acyl transferase".

By these procedures, a partially purified protein preparation is obtained which contains a wax synthase protein having an apparent molecular mass of approximately 57kD. Thus, methods of obtaining wax synthase proteins through purification from seed-plant sources are provided, as well as methods to obtain amino acid sequences of these wax synthase proteins.

In addition, wax synthase proteins from other organisms are provided by methods described herein. For example, a partially purified preparation of an

Acinetobacter wax synthase is obtained, wherein the wax synthase activity is discovered to be associated with an approximately 45kD peptide band. Similarly, a wax synthase protein preparation from *Euglena gracilis* is provided, wherein a 41kD peptide is associated with wax synthase activity.

In a different aspect of this invention, nucleic acid sequences associated with a wax synthase of this invention are considered. Methods are described whereby these sequences may be identified and obtained from the amino acid sequences of the wax synthase proteins of this invention. Uses of the structural gene sequences for isolation of other wax synthase sequences, as well as in recombinant constructs for transcription of wax synthase nucleic acid sequences and/or expression of wax synthase proteins in host cells are described. Uses of other nucleic acid sequences associated with wax synthase protein are also considered, such as the use of 5' and 3' noncoding regions.

In yet a different aspect of this invention, cells containing recombinant constructs coding for sense and antisense wax synthase sequences are considered. In particular, cells which contain the preferred acyl-CoA substrates of a jojoba wax synthase, such as those cells in embryos of *Brassica* plants, are considered.

In addition, cells containing the wax synthase protein of this invention as the result of expression from the recombinant constructs of this invention are considered, and a method of producing a wax synthase in a host cell is provided. Accordingly, a wax synthase protein that is recovered as the result of expression of that protein in a host cell is also considered in this invention.

Further, it may be recognized that the wax synthases of this invention may find application in the production of wax esters in such host cells which contain fatty acyl and fatty alcohol substrates of the wax synthase. Such host cells may exist in nature or be obtained by transformation with nucleic acid constructs which encode a fatty acyl

reductase. Fatty acyl reductase, or "reductase", is active in catalyzing the reduction of a fatty acyl group to the corresponding alcohol. Co-pending US patent applications 07/659,975 and 07/767,251, which are hereby incorporated by reference, are directed to such reductase proteins. This information is also provided in published PCT patent application WO 92/14816. In addition, other sources of wax synthase proteins are described herein which are also desirable sources of reductase proteins.

Especially considered in this aspect of the invention, are plant cells which contain the preferred alcohol substrates of a jojoba wax synthase described herein. A method of providing plant cells with such alcohol substrates is considered wherein said cells are transformed with recombinant nucleic acid constructs which encode a fatty acyl reductase nucleic acid sequence. Thus, plant hosts which do not normally contain significant amounts of the alcohol substrates utilized by wax synthase, may be transformed with a reductase construct such that the alcohols are produced. In this manner, the fatty acyl groups present in the host cell will also provide the source of fatty alcohol substrate utilized by wax synthase in the synthesis of wax esters. Depending on the specificities of the wax synthase and reductase proteins, one recognizes that in this manner, plant cells may be obtained which produce a variety of desirable wax ester products. Such products will have different properties depending on the chain length and degree of saturation of the fatty alcohol and fatty acyl groups. Thus, the wax ester products produced according to the methods herein may be recovered from the host cells and are also considered in this invention.

DETAILED DESCRIPTION OF THE INVENTION

A fatty acyl-CoA: fatty alcohol acyltransferase of this invention includes any sequence of amino acids, such as protein, polypeptide or peptide fragment, which is active in catalyzing the esterification of a fatty alcohol

by a fatty acyl group to produce a wax ester. The acyl-CoA: alcohol acyltransferase of this invention is also referred to hereafter as "wax synthase".

Although typically referred to as an acyl-CoA: alcohol acyltransferase, the wax synthases of this invention may demonstrate activity towards a variety of acyl substrates, including fatty acyl-CoA and fatty acyl-ACP molecules. In addition, both the acyl and alcohol substrates acted upon by the wax synthase may have varying carbon chain lengths and degrees of saturation, although the wax synthase may demonstrate preferential activity towards certain molecules.

Many different organisms produce wax esters from alcohol and acyl substrates and are desirable sources of a wax synthase protein of this invention. For example, plants produce epidermal, or cuticular wax (Kolattukudy (1980) in *The Biochemistry of Plants* (Stumpf, P.K. and Conn, E.E., eds.) *Vol.4*, p. 571-645), and the desert shrub, jojoba, produces a seed storage wax (Ohlrogge *et al.* (*Lipids* (1978) *13*:203-210). Wax synthesis has also been observed in various species of bacteria, such as *Acinetobacter* (Fixter *et al.* (1986) *J. Gen. Microbiol. 132*:3147-3157) and *Micrococcus* (Lloyd (1987) *Microbios 52*:29-37), and by the unicellular orgnanism, *Euglena* (Khan and Kolattukudy (1975) *Arch. Biochem. Biophys. 170*:400-408). In addition, wax production and wax synthase activity have been reported in microsomal preparations from bovine meibomian glands (Kolattukudy *et al.* (1986) *J. Lipid Res. 27*:404-411), avian uropygial glands, and various insect and marine organisms. Consequently, many different wax esters which will have various properties may be produced by the wax synthases of this invention, and the activity of the enzyme and type of wax ester produced may depend upon the available substrate or the substrate specificity of the particular wax synthase of interest.

To obtain a reliable source of a wax synthase protein for use in esterification reactions, it is desirable to isolate nucleic acid sequences associated with the wax

synthase such that these sequences may be cloned into host cells for the production of the wax synthase enzyme. For example, one may clone nucleic acid sequences encoding a wax synthase protein into vectors for expression in *E. coli* cells to provide a ready source of the wax synthase protein. The wax synthase protein so produced may also be used to raise antibodies against wax synthase proteins for use in identification and purification of related wax synthase proteins from various sources, especially from plants. In addition, further study of the wax synthase protein may lead to site-specific mutagenesis reactions to further characterize and improve its catalytic properties or to alter its fatty alcohol or fatty acyl substrate specificity. A wax synthase with altered substrate specificity may find application in conjunction with other FAS enzymes.

Prior to the instant invention, amino acid sequences of wax synthase proteins were not known. Thus, in order to obtain the nucleic acid sequences associated with wax synthase, it was necessary to first purify the protein from an available source and determine at least partial amino acid sequence so that appropriate probes useful for isolation of wax synthase nucleic acid sequences could be prepared.

The desert shrub, *Simmondsia chinensis* (jojoba) was identified as a source of a candidate wax synthase protein. Initial studies reveal that the jojoba wax synthase is an integral membrane protein and hydrophobic in nature. In general, membrane associated proteins are difficult to purify as they tend to lose enzymatic activity when they are solubilized, i.e. separated from the membrane environment in which they normally function. Techniques that have been used to solubilize integral membrane proteins include addition of detergents or organic solvents to a preparation of a suitable membrane fraction. Further conventional purification techniques, such as precipitation, ion-exchange, gel-filtration and affinity chromatography may then be utilized, assuming the desired

protein still retains functional activity that can be measured using a specific enzymatic assay.

Typically, as a first step towards obtaining a solubilized membrane protein, a microsomal membrane preparation which comprises wax synthase activity is desired. Standard microsomal membrane preparations utilize differential centrifugation of a cell-free homogenate (CFH) to yield a membrane fraction which is free of whole cells, nuclei and soluble protein. (See, for example Mooré *et al.* (1987) *Biological Membranes: A Practical Approach*, pp. 37-72, eds. Finalay and Evans.) With oilseeds, initial centrifugation steps typically yield a pellet, supernatant and a floating fat pad, and microsomal membranes may then be recovered by further centrifugation of the supernatant.

A protocol is described in co-pending USSN 07/659,975, filed February 22, 1991, whereby a jojoba membrane fraction was obtained with good recovery of enzyme activity associated with fatty acyl reductase, another enzyme involved in the formation of wax esters in jojoba. The method also provides membrane fractions having wax synthase activity as described in detail in the examples which follow. Other procedures are known to those in the art and may be utilized to obtain similar membrane preparations. In addition, methods to assay for wax synthase activity in such preparations are described in Example 1.

A critical stage for further enzyme characterization and purification is that of obtaining solubilized wax synthase protein that is separated from its native lipid bilayer membrane environment, but retains substantial amounts of measurable wax synthase enzymatic activity. The removal of integral membrane proteins from the lipid bilayer is typically accomplished using amphiphilic detergents in aqueous solution, although organic solvents have also been used in a few cases. Many different detergents and methods of solubilization of membrane proteins are known to those skilled in the art, and are also reviewed by Neugebauer (*Methods Enzymol.* (1990)

182:239-253) and Hjelmiland (*Methods Enzymol.* (1990) *182*:253-264).

Often, detergents which are used to solubilize membrane proteins are found to inhibit the enzymatic activity of a desired protein. Several detergents were tested for solubilization of jojoba wax synthase, including CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), which was demonstrated in copending USSN 07/659,975, to be useful in purification of a fatty acyl reductase from jojoba. All were found to inhibit wax synthase enzymatic activity. Although strong inhibition by CHAPS was observed at concentrations above the CMC, it was found that addition of phospholipids, such as L-phosphatidyl choline, and adjustment of the CHAPS concentration from 0.75% to 0.3%, i.e. to below the CMC, results in reconstitution of a portion of the wax synthase activity. The primary requirement for reconstitution of wax synthase activity is the presence of phospholipids during the removal or dilution of the detergent, so that the wax synthase protein is incorporated into phospholipid vesicles. This differs from the protocol developed for reconstitution of jojoba reductase activity, which does not require addition of phospholipids. Thus, if phospholipids are present in a wax synthase preparation, such as that from a microsomal membrane fraction, activity may be detected simply by removal or dilution of detergent. However, in further purified wax synthase preparations, phospholipids must be added to detect activity. If high levels, ie. greater than approximately 2% (w/v), of phospholipids are used, wax synthase activity may be restored by simple dilution of the detergent used for solubilization. A method to reconstitute and assay wax synthase activity in solubilized wax synthase preparations is described in Example 1.

A protocol for solubilizing jojoba wax synthase activity utilizing the detergent CHAPS is described in Example 4. Yields of approximately 15% of the wax synthase activity from the microsomal membrane preparation are

obtained. Similarly, studies of reversibility of apparent wax synthase inhibition by other detergents may be conducted to identify other useful detergents for solubilization of the jojoba or other candidate wax synthases. As the percentage of wax synthase activity which is solubilized from the microsomal membrane preparation is small, techniques may be developed to increase the percentage of wax synthase obtained in a solubilized form. However, the proportion of solubilized wax synthase obtained by the described methods is sufficient to permit further purification, characterization and sequencing of the wax synthase as described below.

Having obtained solubilized wax synthase protein, it can be seen that further experiments to characterize the enzyme as to substrate specificity, cofactor requirements and possible activity inhibiting agents may now be conducted. For example, it has been found that the jojoba wax synthase of this invention has a broad range of acyl substrates, including acyl-ACP and acyl-CoA molecules. In addition, the acyl and fatty alcohol substrates may have a broad size range with respect to carbon chain length. For example, activity was tested using substrates having carbon chain lengths of from C12 to C24, and all were shown to be utilized by the enzyme. In addition, activity was shown with fatty acyl and fatty alcohols having varying degrees of unsaturation.

A procedure which has proved very useful for further characterization of the wax synthase of this invention is that of specifically labeling the wax synthase protein. For example, it was discovered that membrane preparations having wax synthase activity may be incubated with radiolabeled palmitoyl-CoA, a substrate of the wax synthase enzyme, such that a radiolabeled peptide band of apparent molecular mass of approximately 57kD is detected by SDS polyacrylamide electrophoresis (PAGE) and subsequent autoradiography. In addition, solubilized wax synthase protein, which no longer demonstrates enzymatic activity, may be similarly labeled to provide a convenient method to

track the wax synthase protein through further purification steps. Details of these labeling procedures are described in Example 2.

Thus, preparations comprising wax synthase activity of this invention may be subjected to further techniques, such as SDS polyacrylamide gel electrophoresis (PAGE) and subsequent staining, or radiolabeling with palmitoyl-CoA, followed by SDS PAGE and subsequent autoradiography. In this manner, it is verified that an approximately 57kD protein is present in these preparations and that the staining intensity of this protein corresponds to levels of wax synthase activity. When palmitoyl-CoA radiolabeling is conducted, SDS PAGE and autoradiography confirm that the labeled band tracks with wax synthase activity. Experiments which verify that the 57kD peptide band tracks with wax synthase activity in fractions from size exclusion, affinity and reactive dye chromatography, are described in the following examples.

In addition, chromatography techniques may be utilized to provide enriched preparations of plant wax synthase. One such purification step involves chromatography over an immobilized reactive dye matrix, such as the Cibacron Blue F3GA (Blue A) used in this invention. The jojoba wax synthase activity binds to such a column when loaded in a buffer containing approximately 0.4M NaCl, while greater than approximately 85% of other protein passes through or is removed in subsequent washes. As described in copending application USSN 07/767,251, reductase activity is also bound to the Blue A column under such conditions. It is demonstrated herein that approximately 20% of the wax synthase activity loaded to a Blue A column can be recovered by elution. A small portion of this wax synthase activity is eluted with a 1.0M NaCl buffer wash, which also contains the majority of the reductase activity which is recovered from this column. The majority of the recovered wax synthase activity is obtained by elution with 1.5M NaCl buffer, wash which also contains a small proportion of the reductase activity. Thus, the majority of the recoverable

wax synthase activity is separated from the majority of the reductase protein, although the major proteins present in the preparation other than the 57kD wax synthase, are the 56 and 54kD reductase proteins.

Further studies of the wax synthase protein following Blue A chromatography indicate that the wax synthase protein may be undergoing aggregation on this column. For example, size exclusion chromatography of Blue A fractions having wax synthase activity on Superose 12 (Pharmacia), results in elution of the majority of wax synthase activity in the void fractions of the column (exclusion limit approximately 5 million daltons), indicating that the wax synthase is in an aggregated form. Importantly, a small fraction (~5%) of the wax synthase activity is detected in the retained fractions, and the size of this peak activity is estimated at ~55kD by comparison to protein standards. This provides additional evidence that the 57kD labeled band is wax synthase, and also demonstrates that wax synthase activity is provided by a single polypeptide.

Using such labeling and purification techniques, the jojoba wax synthase protein can be recovered as a substantially purified protein preparation and the amino acid sequence can be obtained. Similarly, due to the hydrophobic nature of the fatty alcohol substrates of wax synthase enzymes, other wax synthases would also be predicted to be associated with membranes in their native cells, and thus purification techniques described herein for jojoba wax synthase, may also be useful in recovery of purified preparation of other wax synthase proteins.

For example, *Euglena gracilis* produces waxes through the enzymatic actions of a fatty acyl-CoA reductase and a fatty acyl-CoA alcohol transacylase, or wax synthase. Typically, waxes having carbon chain lengths ranging from 24-32 are detected in this organism. As described above for jojoba, the *Euglena* wax synthase enzyme may be solubilized using a CHAPS/NaCl solution, and a partially purified wax synthase preparation is obtained by Blue A

chromatography. In this manner, a 41kD peptide band associated with wax synthase activity is identified.

Acinetobacter species are also known to produce wax ester compositions, although the mechanism is not well defined. As described herein a fatty acyl-CoA alcohol transacylase, or wax synthase activity is detected in *Acinetobacter* species. The wax synthase activity is solubilized in CHAPS/NaCl, enriched by Blue A column chromatography and may be further purified using such techniques as size exclusion chromatography. By these methods, an approximately 45kD peptide band associated with wax synthase activity is obtained in a partially purified preparation.

Although the hydrophobic nature of these wax synthase proteins presents challenges to purification, recovery of substantially purified protein can be accomplished using a variety of methods. For example, a preparative electrophoresis apparatus which utilizes continuous elution electrophoresis process, may be used to purify the 57kD wax synthase protein obtained from the Blue A column. In this manner, gel fractions may be identified which contain the wax synthase protein in a substantially pure form in a liquid solution. The wax synthase protein sample may then be dialyzed, if necessary, and concentrated to provide a convenient protein source for amino acid sequencing techniques.

Alternatively, polyacrylamide gels may be run and the proteins transferred to a membrane support, such as nitrocellulose or polyvinylidenedifluoride (PVDF). The sections of these membranes which contain the wax synthase protein may then be obtained such that the wax synthase is substantially free of other proteins. The wax synthase protein may then be removed from the membranes and further manipulated such that the amino acid sequences is determined. As the wax synthase protein of this invention, transfers poorly to nitrocellulose membranes, PVDF is preferred for sequencing methods.

Thus, amino acid sequence of wax synthase is determined by sequencing N-terminal amino acid regions from whole protein or by preparing fragments of the desired protein by digestion with the chemical cyanogen bromide, or alternatively by enzymatic cleavage using proteases. Examples of proteases which may be useful include trypsin, and endoproteinases lysC, gluC, AspN and argC. The wax synthase peptides obtained in this manner may then be purified and sequenced in accordance with methods familiar to those skilled in the art. These peptide sequences may then be used in gene isolation techniques, including PCR methods and cDNA and genomic library screening.

Further experiments to confirm the identity of the wax synthase may also be desirable, such as expression of the protein in *E. coli*. The wax synthase may then act on fatty acyl and fatty alcohol substrates in such cells to produce wax esters which may be detected by various anlaytical methods. If the host cells do not contain the alcohol substrate of the wax synthase, activity may be verified by assaying cell extracts. Alternatively, wax synthase protein may be prepared by *in vitro* translation using wax synthase nucleic acid sequences and commercially available translation kits. Addition of microsomal membrane preparations to the *in vitro* translation sample may be necessary to obtain active wax synthase protein if membrane insertion is critical to activity. Other testing may include immunological assays, whereby antibodies specific for the candidate protein are prepared and found to inhibit wax synthase activity in protein preparations.

Thus, it is desirable to isolate nucleic acid sequences using amino acid sequences determined for the proteins associated with wax synthase activity, both to confirm the identity of an wax synthase protein and to provide for transcription of the sequences and/or expression of the protein in host cells, either prokaryotic or eukaryotic.

As the wax synthase is a membrane bound protein, it may be desirable to express a candidate protein in a plant

cell in order to verify the activity. Electroporation or bombardment of plant tissue for transient expression may be useful for this purpose. Ultimately, stable plant expression in a plant which produces substrates recognized by this enzyme is desired. If a plant targeted for transformation with wax synthase sequences does not naturally contain the fatty alcohol and fatty acyl ester substrates of this enzyme, a plant extract may be prepared and assayed for wax synthase activity by adding substrates of the wax synthase to the extract. Constructs and methods for transformation of plant hosts with wax synthase sequences are discussed in more detail below.

The nucleic acids of this invention may be genomic or cDNA and may be isolated from cDNA or genomic libraries or directly from isolated plant DNA. Methods of obtaining gene sequences once a protein is purified and/or amino acid sequence of the protein is obtained are known to those skilled in the art.

For example, antibodies may be raised to the isolated protein and used to screen expression libraries, thus identifying clones which are producing the plant wax synthase protein or an antigenic fragment thereof. Alternatively, oligonucleotides may be synthesized from the amino acid sequences and used in isolation of nucleic acid sequences. The oligonucleotides may be useful in PCR to generate a nucleic acid fragment, which may then be used to screen cDNA or genomic libraries. In a different approach, the oligonucleotides may be used directly to analyze Northern or Southern blots in order to identify useful probes and hybridization conditions under which these oligonucleotides may be used to screen cDNA or genomic libraries.

Wax synthase nucleic acid sequences of this invention include those corresponding to the jojoba wax synthase protein, as well as sequences obtainable from the jojoba protein or nucleic acid sequences. By "corresponding" is meant nucleic acid sequences, either DNA or RNA, including those which encode jojoba wax synthase protein or a portion

thereof, regulatory sequences found 5' or 3' to said encoding sequences which direct the transcription or transcription and translation (expression) of the wax synthase in jojoba embryos, intron sequences not present in the cDNA, as well as sequences encoding any leader or signal peptide of a precursor wax synthase protein that may be required for insertion into the endoplasmic reticulum membrane, but is not found in the mature wax synthase enzyme.

By sequences "obtainable" from the jojoba sequence or protein, is intended any nucleic acid sequences associated with a desired wax synthase protein that may be synthesized from the jojoba wax synthase amino acid sequence, or alternatively identified in a different organism, and isolated using as probes jojoba wax synthase nucleic acid sequences or antibodies prepared against the jojoba wax synthase protein. In this manner, it can be seen that sequences of these other wax synthases may similarly be used to isolate nucleic acid sequences associated with wax synthase proteins from additional sources.

For isolation of nucleic acid sequences, cDNA or genomic libraries may be prepared using plasmid or viral vectors and techniques well known to those skilled in the art. Useful nucleic acid hybridization and immunological methods that may be used to screen for the desired sequences are also well known to those in the art and are provided, for example in Maniatis, *et al.* (*Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Typically, a sequence obtainable from the use of nucleic acid probes will show 60-70% sequence identity between the target sequence and the given sequence encoding a wax synthase enzyme of interest. However, lengthy sequences with as little as 50-60% sequence identity may also be obtained. The nucleic acid probes may be a lengthy fragment of the nucleic acid sequence, or may also be a shorter, oligonucleotide probe. When longer nucleic acid fragments are employed as probes (greater than about 100

bp), one may screen at lower stringencies in order to obtain sequences from the target sample which have 20-50% deviation (i.e., 50-80 sequence homology) from the sequences used as probe. Oligonucleotide probes can be considerably shorter than the entire nucleic acid sequence encoding a wax synthase enzyme, but should be at least about 10, preferably at least about 15, and more preferably at least about 20 nucleotides. A higher degree of sequence identity is desired when shorter regions are used as opposed to longer regions. It may thus be desirable to identify enzyme active sites where amino acid sequence identity is high to design oligonucleotide probes for detecting homologous genes.

To determine if a related gene may be isolated by hybridization with a given sequence, the sequence is labeled to allow detection, typically using radioactivity, although other methods are available. The labeled probe is added to a hybridization solution, and incubated with filters containing the desired nucleic acids, either Northern or Southern blots (to screen desired sources for homology), or the filters containing cDNA or genomic clones to be screened. Hybridization and washing conditions may be varied to optimize the hybridization of the probe to the sequences of interest. Lower temperatures and higher salt concentrations allow for hybridization of more distantly related sequences (low stringency). If background hybridization is a problem under low stringency conditions, the temperature can be raised either in the hybridization or washing steps and/or salt content lowered to improve detection of the specific hybridizing sequence. Hybridization and washing temperatures can be adjusted based on the estimated melting temperature of the probe as discussed in Beltz, *et al.* (*Methods in Enzymology* (1983) *100*:266-285).

A useful probe and appropriate hybridization and washing conditions having been identified as described above, cDNA or genomic libraries are screened using the labeled sequences and optimized conditions. The libraries

are first plated onto a solid agar medium, and the DNA lifted to an appropriate membrane, usually nitrocellulose or nylon filters. These filters are then hybridized with the labeled probe and washed as discussed above to identify clones containing the related sequences.

For immunological screening, antibodies to the jojoba wax synthase can be prepared by injecting rabbits or mice (or other appropriate small mammals) with the purified protein. Methods of preparing antibodies are well known to those in the art, and companies which specialize in antibody production are also available. Either monoclonal or polyclonal antibodies can be produced, although typically polyclonal antibodies are more useful for gene isolation.

To screen desired plant species, Western analysis is conducted to determine that a related protein is present in a crude extract of the desired plant species, that cross-reacts with the antibodies to the jojoba wax synthase. This is accomplished by immobilization of the plant extract proteins on a membrane, usually nitrocellulose, following electrophoresis, and incubation with the antibody. Many different systems for detection of the antibody/protein complex on the nitrocellulose filters are available, including radiolabeling of the antibody and second antibody/enzyme conjugate systems. Some of the available systems have been described by Oberfelder (*Focus* (1989) BRL/Life Technologies, Inc. *11*:1-5). If initial experiments fail to detect a related protein, other detection systems and blocking agents may be utilized. When cross-reactivity is observed, genes encoding the related proteins can be isolated by screening expression libraries representing the desired plant species. Expression libraries can be constructed in a variety of commercially available vectors, including lambda gt11, as described in Maniatis, *et al.* (*supra*).

The clones identified as described above using DNA hybridization or immunological screening techniques are then purified and the DNA isolated and analyzed using known

techniques. In this manner, it is verified that the clones encode a related wax synthase protein. Other wax synthases may be obtained through the use of the "new" wax synthase in the same manner as the jojoba wax synthase was used.

It will be recognized by one of ordinary skill in the art that wax synthase nucleic acid sequences of this invention may be modified using standard techniques of site specific mutation or PCR, or modification of the sequence may be accomplished in producing a synthetic nucleic acid sequence. These modified sequences are also considered wax synthase nucleic acid sequence of this invention. For example, wobble positions in codons may be changed such that the nucleic acid sequence encodes the same amino acid sequence, or alternatively, codons can be altered such that conservative amino acid substitutions result. In either case, the peptide or protein maintains the desired enzymatic activity and is thus considered part of the instant invention.

A nucleic acid sequence of a wax synthase enzyme of this invention may be a DNA or RNA sequence, derived from genomic DNA, cDNA, mRNA, or may be synthesized in whole or in part. The gene sequences may be cloned, for example, by isolating genomic DNA from an appropriate source, and amplifying and cloning the sequence of interest using a polymerase chain reaction (PCR). Alternatively, the gene sequences may be synthesized, either completely or in part, especially where it is desirable to provide plant-preferred sequences. Thus, all or a portion of the desired structural gene (that portion of the gene which encodes the wax synthase protein) may be synthesized using codons preferred by a selected host. Host-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a desired host species.

The nucleic acid sequences associated with wax synthase proteins will find many uses. For example, recombinant constructs can be prepared which can be used as probes or will provide for expression of the wax synthase

protein in host cells. Depending upon the intended use, the constructs may contain the sequence which encodes the entire wax synthase, or a portion thereof. For example, critical regions of the wax synthase, such as an active site may be identified. Further constructs containing only a portion of the wax synthase sequence which encodes the amino acids necessary for a desired wax synthase activity may thus be prepared.

Useful systems for expression of the wax synthase sequences of this invention include prokaryotic cells, such as *E. coli*, yeast cells, and plant cells, both vascular and nonvascular plant cells being desired hosts. In this manner, the wax synthase protein may be produced to allow further studies, such as site-specific mutagenesis of encoding sequences to analyze the effects of specific mutations on reactive properties of the wax synthase protein.

The DNA sequence encoding a wax synthase of this invention may be combined with foreign DNA sequences in a variety of ways. By "foreign" DNA sequences is meant any DNA sequence which is not naturally found joined to the wax synthase sequence, including DNA sequences from the same organism which are not naturally found joined to wax synthase sequences. Both sense and antisense constructs utilizing wax synthase encoding sequences are considered, wherein sense sequence may be used for expression of wax synthase in a host cell, and antisense sequences may be used to decrease the endogenous levels of a homologous wax synthase protein naturally produced by a target organism. In addition, the wax synthase gene sequences of this invention may be employed in a foreign host in conjunction with all or part of the sequences normally associated with the wax synthase, such as regulatory or membrane targeting sequences.

In its component parts, a DNA sequence encoding wax synthase is combined in a recombinant construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting

transcription and translation in a host cell, the nucleic acid sequence encoding wax synthase and a transcription termination region. Depending upon the host, the regulatory regions will vary, and may include regions from viral, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Expression in a microorganism can provide a ready source of the plant enzyme. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *E. coli, B. subtilis, Sacchromyces cerevisiae,* including genes such as beta-galactosidase, T7 polymerase, tryptophan E and the like.

For the most part, the recombinant constructs will involve regulatory regions functional in plants which provide for expression of the wax synthase gene to produce functional wax synthase protein. The open reading frame, coding for the plant wax synthase or a functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region such as the wild-type sequence naturally found 5' upstream to the wax synthase structural gene. Numerous other promoter regions from native plant genes are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, expression of structural gene sequences.

In addition to sequences from native plant genes, other sequences can provide for constitutive gene expression in plants, such as regulatory regions associated with *Agrobacterium* genes, including regions associated with nopaline synthase (*Nos*), mannopine synthase (*Mas*), or octopine synthase (*Ocs*) genes. Also useful are regions which control expression of viral genes, such as the 35S and 19S regions of cauliflower mosaic virus (CaMV). The term constitutive as used herein does not necessarily indicate that a gene is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types, although some variation in abundance is

often detectable. Other useful transcriptional initiation regions preferentially provide for transcription in certain tissues or under certain growth conditions, such as those from napin, seed or leaf ACP, the small subunit of RUBISCO, and the like.

In embodiments wherein the expression of the wax synthase protein is desired in a plant host, the use of all or part of the complete plant wax synthase gene may be desired, namely the 5' upstream non-coding regions (promoter) together with the structural gene sequence and 3' downstream non-coding regions may be employed. If a different promoter is desired, such as a promoter native to the plant host of interest or a modified promoter, i.e., having transcription initiation regions derived from one gene source and translation initiation regions derived from a different gene source or enhanced promoters, such as double 35S CaMV promoters, the sequences may be joined together using standard techniques.

The DNA constructs which provide for wax synthase expression in plants may be employed with a wide variety of plant life, particularly, plants which produce the fatty acyl-CoA substrates of the wax synthase enzyme, such as *Brassica*. Other plants of interest produce desirable fatty acyl substrates, such as medium or long chain fatty acyl molecules, and include but are not limited to rapeseed (Canola varieties), sunflower, safflower, cotton, *Cuphea*, soybean, peanut, coconut and oil palms, and corn.

As to the fatty alcohol substrate of the wax synthase enzyme, other than jojoba, seed plants are not known to produce large quantities of fatty alcohols, although small amounts of this substrate may be available to the wax synthase enzyme. Therefore, in conjunction with the wax synthase constructs of this invention, it is desirable to provide the target host cell with the capability to produce fatty alcohols from the fatty acyl molecules present in the host cells. For example, a plant fatty acyl reductase and methods to provide for expression of the reductase enzymes in plant cells are described in co-pending application USSN

07/767,251. The nucleic acid sequence and translated amino acid sequence of the jojoba reductase is provided in Figure 1. Thus, by providing both the wax synthase and reductase proteins to the host plant cell, wax esters may be produced from the fatty alcohol and fatty acyl substrates.

In addition to the jojoba reductase, reductase enzymes from other organisms may be useful in conjunction with the wax synthases of this invention. Other potential sources of reductase enzymes include *Euglena, Acinetobacter, Micrococus*, certain insects and marine organisms, and specialized mammalian or avian tissues which are known to contain wax esters, such as bovine meibomian glands or ovian uropygial glands. Other potential sources may be identified by their ability to produce fatty alcohols or, if wax synthase is also present, wax esters.

The wax synthase and reductase sequences may be provided during the same transformation event, or alternatively, two different transgenic plant lines, one having wax synthase constructs and the other having reductase constructs may be produced by transformation with the various constructs. These plant lines may then be crossed using known plant breeding techniques to provide wax synthase and reductase containing plants for production of wax ester products.

For applications leading to wax ester production, 5' upstream non-coding regions obtained from genes regulated during seed maturation are desired, especially those preferentially expressed in plant embryo tissue, such as regions derived from ACP and napin regulatory regions. Transcription initiation regions which provide for preferential expression in seed tissue, i.e., which are undetectable in other plant parts, are considered desirable for wax ester production in order to minimize any disruptive or adverse effects of the gene product in other plant parts. Further, the seeds of such plants may be harvested and the lipid reserves of these seeds recovered to provide a ready source of wax esters. Thus, a novel seed product may be produced in oilseed plants which,

absent transformation with wax synthase constructs as described herein, are not known to produce wax esters as a component of their seed lipid reserves.

Such "seed-specific promoters" may be obtained and used in accordance with the teachings of U.S. Serial No. 07/147,781, filed 1/25/88 (now U.S. Serial No. 07/742,834, filed 8/8/81), and U.S. Serial No. 07/494,722 filed on March 16, 1990 having a title "Novel Sequences Preferentially Expressed In Early Seed Development and Methods Related Thereto", all of which copending applications are incorporated herein by reference.

Regulatory transcription termination regions may be provided in recombinant constructs of this invention as well. Transcription termination regions may be provided by the DNA sequence encoding the plant wax synthase or a convenient transcription termination region derived from a different gene source, especially the transcription termination region which is naturally associated with the transcription initiation region. The transcript termination region will contain at least about 0.5kb, preferably about 1-3kb of sequence 3' to the structural gene from which the termination region is derived.

Depending on the method for introducing the DNA expression constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to dicotyledyons and monocotyledons species alike and will be readily applicable to new and/or improved transformation and regeneration techniques.

In developing the recombinant construct, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector which is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors exist that have been described in the literature. After each cloning, the plasmid may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, insertion, resection, etc., so as to tailor the components of the desired sequence. Once the construct has

been completed, it may then be transferred to an appropriate vector for further manipulation in accordance with the manner of transformation of the host cell.

Normally, included with the recombinant construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Similarly, genes encoding enzymes providing for production of a compound identifiable by color change, such as GUS, or luminescence, such as luciferase are useful. Depending upon the number of different host species the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

In addition to the sequences providing for transcription of wax synthase sequences, the DNA constructs of this invention may also provide for expression of an additional gene or genes, whose protein product may act in conjunction with the wax synthase to produce a valuable end product. For example, as discussed above, DNA constructs which provide for expression of wax synthase and a fatty acyl reductase so that wax esters may produced in transformed hosts, are considered in this invention. Furthermore, production of different wax esters having varying carbon chain lengths and degrees of saturation is desired and may be provided by transforming host plants having fatty alcohol or fatty acy substrates of varying chain lengths. Such plants may be provided, for example, by methods described in the published international patent application number PCT WO 91/16421, which describes various thioesterase genes and methods of using such genes to produce fatty acyl substrates having varying chain lengths in transformed plant hosts.

Furthermore, to optimize the production of wax esters in oilseed plant hosts, one may wish to decrease the

production of the triacylglyceride oils that are normally produced in the seeds of such plants. One method to accomplish this is to antisense a gene critical to this process, but not necessary for the production of wax esters. Such gene targets include diacylglycerol acyltransferase, and other enzymes which catalyse the synthesis of triacylglycerol. Additionally, it may be desirable to provide the oilseed plants with enzymes which may be used to degrade wax esters as a nutrient source, such as may be isolated from jojoba or various other wax producing organisms. In this manner, maximal production of wax esters in seed plant hosts may be achieved.

The wax esters produced in the methods described herein may be harvested using techniques for wax extraction from jojoba or by various production methods used to obtain oil products from various oilseed crops. The waxes thus obtained will find application in many industries, including pharmaceuticals, cosmetics, detergents, plastics, and lubricants. Applications will vary depending on the chain length and degree of saturation of the wax ester components. For example, long chain waxes having a double band in each of the carbon chains are liquid at room temperature, whereas waxes having saturated carbon chain components, may be solid at room temperature, especially if the saturated carbon chains are longer carbon chains.

The method of transformation is not critical to the instant invention; various methods of plant transformation are currently available. As newer methods are available to transform crops, they may be directly applied hereunder. For example, many plant species naturally susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. Other sequences useful in providing for transfer of nucleic acid sequences to host plant cells may be derived from plant pathogenic viruses or plant transposable elements. In addition, techniques of microinjection, DNA particle bombardment, electroporation

have been developed which allow for the transformation of various monocot and dicot plant species.

When *Agrobacterium* is utilized for plant transformation, it may be desirable to have the desired nucleic acid sequences bordered on one or both ends by T-DNA, in particular the left and right border regions, and more particularly, at least the right border region. These border regions may also be useful when other methods of transformation are employed.

Where *Agrobacterium* or *Rhizogenes* sequences are utilized for plant transformation, a vector may be used which may be introduced into an *Agrobacterium* host for homologous recombination with the T-DNA on the Ti- or Ri-plasmid present in the host. The Ti- or Ri- containing the T-DNA for recombination may be armed (capable of causing gall formation), or disarmed (incapable of causing gall formation), the latter being permissible so long as a functional complement of the *vir* genes, which encode trans-acting factors necessary for transfer of DNA to plant host cells, is present in the transformed *Agrobacterium* host. Using an armed *Agrobacterium* strain can result in a mixture of normal plant cells, some of which contain the desired nucleic acid sequences, and plant cells capable of gall formation due to the presence of tumor formation genes. Cells containing the desired nucleic acid sequences, but lacking tumor genes can be selected from the mixture such that normal transgenic plants may be obtained.

In a preferred method where *Agrobacterium* is used as the vehicle for transforming host plant cells, the expression or transcription construct bordered by the T-DNA border region(s) will be inserted into a broad host range vector capable of replication in *E. coli* and *Agrobacterium*, there being broad host range vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, Ditta, *et al.*, (*Proc. Nat. Acad. Sci., U.S.A.* (1980) 77:7347-7351) and EPA 0 120 515, which are incorporated herein by reference. Alternatively, one may insert the sequences to be expressed in plant cells into a

vector containing separate replication sequences, one of which stabilizes the vector in *E. coli*, and the other in *Agrobacterium*. See, for example, McBride and Summerfelt (*Plant Mol. Biol.* (1990) *14*:269-276), wherein the pRiHRI (Jouanin, *et al.*, *Mol. Gen. Genet.* (1985) 201:370-374) origin of replication is utilized and provides for added stability of the plant expression vectors in host *Agrobacterium* cells.

Utilizing vectors such as those described above, which can replicate in *Agrobacterium* is preferred. In this manner, recombination of plasmids is not required and the host *Agrobacterium vir* regions can supply trans-acting factors required for transfer of the T-DNA bordered sequences to plant host cells. For transformation of *Brassica* cells, *Agrobacterium* transformation methods may be used. One such method is described, for example, by Radke *et al.* (*Theor. Appl. Genet.* (1988) *75*:685-694).

In addition

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included for purposes of illustration only and are not intended to limit the invention unless so stated.

EXAMPLES

Example 1 - Wax synthase Assays

Methods to assay for wax synthase activity in microsomal membrane preparations or solubilized protein preparations are described.

A. Radiolabeled Material

The substrate generally used in the wax synthase assays, [1-^{14}C]palmitoyl-CoA, is purchased from Amersham (Arlington Heights, IL). Other chain length substrates were synthesized in order to perform chain length specification studies. Long chain [1-^{14}C] fatty acids (specific activity 51-56 Ci/mole), namely 11-cis-eicosenoic acid, 13-cis-docosenoic acid and 15-cis-tetracosenoic acid

are prepared by the reaction of potassium [^{14}C]cyanide with the corresponding alcohol mesylate, followed by the base hydrolysis of the alcohol nitrile to the free fatty acid. The free fatty acids are converted to their methyl esters with ethereal diazomethane, and purified by preparative silver nitrate thin layer chromatography (TLC). The fatty acid methyl esters are hydrolyzed back to the free fatty acids. Radiochemical purity is assessed by three TLC methods: normal phase silica TLC, silver nitrate TLC, and C18 reversed phase TLC. Radiochemical purity as measured by these methods was 92-98%. Long chain [1-^{14}C] acyl-CoAs are prepared from the corresponding [1-^{14}C] free fatty acids by the method of Young and Lynen (*J. Bio. Chem.* (1969) *244*:377), to a specific activity of 10Ci/mole. [1-^{14}C]hexadecanal is prepared by the dichromate oxidation of [1-^{14}C]hexadecan-1-ol, according to a micro-scale modification of the method of Pletcher and Tate (*Tet. Lett.* (1978) 1601-1602). The product is purified by preparative silica TLC, and stored as a hexane solution at -70°C until use.

B. Assay for Wax synthase Activity in a Microsomal Membrane

Preparation

Wax synthase activity in a microsomal membrane preparation is measured by incubation of 40μM [1-^{14}C]acyl-CoA (usually palmitoyl-CoA, sp. act. 5.1-5.6 mCi/mmol) and 200μM oleyl alcohol with the sample to be assayed in a total volume of 0.25ml. The incubation mixture also contains 20% w/v glycerol, 1mM DTT, 0.5M NaCl and is buffered with 25mM HEPES (4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid). HEPES, here and as referred to hereafter is added from a 1M stock solution adjusted to pH 7.5.

A substrate mixture is prepared in a glass vial, with oleyl alcohol being added immediately before use, and is added to samples. Incubation is carried out at 30°C for one hour. The assay is terminated by placing the assay tube on ice and immediately adding 0.25ml

isopropanol:acetic acid (4:1 v/v). Unlabeled wax esters (0.1mg) and oleyl alcohol (0.1mg) are added as carriers. The [^{14}C] lipids are extracted by the scaled-down protocol of Hara and Radin (*Anal. Biochem.* (1978) *90*:420). Four ml of hexane/isopropanol (3:2, v/v) is added to the terminated assay. The sample is vortexed, 2ml of aqueous sodium sulphate solution (6.6% w/v) is added, and the sample is again vortexed.

C. Assay for Solubilized Wax synthase Activity

For assaying solubilized wax synthase activity, reconstitution of the protein is required. Reconstitution is achieved by the addition of phospholipids (Sigam P-3644, ~40% L-phosphatidyl choline) to the 0.75% CHAPS-solubilized sample at a concentration of 2.5mg/ml, followed by dilution of the detergent to 0.3%, below the CMC. Reconstitution of activity is presumed to be based on the incorporation of wax synthase into the phospholipid vesicles. It is recognized that the amount of wax synthase activity detected after their reconstitution can be influenced by many factors (e.g., the phospholipid to protein ratio and the physical state of the wax synthase protein (e.g. aggregate or dispersed).

D. Analysis of Assay Products

For analyzing the products of either the microsomal membrane preparation wax synthase assay or the solubilized wax synthase assay, two protocols have been developed. One protocol, described below as "extensive assay" is more time-consuming, but yields more highly quantitative results. The other protocol, described below as "quick assay" also provides a measure of wax synthase activity, but is faster, more convenient and less quantitative.

1. *Extensive Analysis*: Following addition of the sodium sulphate and vortexing the sample, the upper organic phase is removed and the lower aqueous phase is washed with 4ml hexane/isopropanol (7:2 v/v). The organic phases are pooled and evaporated to dryness under nitrogen. The lipid residue is resuspended in a small volume of hexane, and an aliquot is assayed for radioactivity by liquid

scintillation counting. The remainder of the sample can be used for TLC analysis of the labeled classes and thereby give a measure of total wax produced.

For lipid class analysis the sample is applied to a silica TLC plate, and the plate is developed in hexane/diethyl ether/acetic acid (80:20:1 v/v/v). The distribution of radioactivity between the lipid classes, largely wax esters, free fatty acids, fatty alcohols, and polar lipids at the origin, is measured using an AMBIS radioanalytic imaging system (AMBIS Systems Inc., San Diego, CA). If necessary the individual lipid classes can be recovered from the TLC plate for further analysis. Reversed-phase TLC systems using C18 plates developed in methanol have also been used for the analysis.

2. *Quick Analysis*: Following addition of the sodium sulfate and vortexing the sample, a known percentage of the organic phase is removed and counted via liquid scintillation counting. This calculation is used to estimate the total counts in the organic phase. Another portion of the organic phase is then removed, dryed down under nitrogen, redissolved in hexane and spotted on TLC plates and developed and scanned as described for the detailed assay. In this manner the percentage of the total counts which are incorporated into wax is determined.

Example 2 - Radiolabeling Wax Synthase Protein

Radiolabeled [1-^{14}C]palmitoyl-CoA (Amersham) is added to a wax synthase preparation, either solubilized or a microsomal membrane fraction, in the ratio of 5µl of label to 40µl protein sample. The sample is incubated at room temperature for at least 15 minutes prior to further treatment. For SDS-PAGE analysis the sample is treated directly with SDS sample buffer and loaded onto gels for electrophoresis.

Example 3 - Further Studies to Characterize Wax Synthase Activity

A. Seed Development and Wax Synthase Activity Profiles

Embryo development was tracked over two summers on five plants in Davis, CA. Embryo fresh and dry weights were found to increase at a fairly steady rate from about day 80 to about day 130. Lipid extractions reveal that when the embryo fresh weight reaches about 300mg (about day 80), the ratio of lipid weight to dry weight reaches the maximum level of 50%.

Wax synthase activity was measured in developing embryos as described in Example 1. As the jojoba seed coats were determined to be the source of an inhibiting factor(s), the seed coats were removed prior to freezing the embryos in liquid nitrogen for storage at -70°C.

Development profiles for wax synthase activities as measured in either a cell free homogenate or a membrane fraction, indicate a large induction in activity which peaks at approximately 110-115 days after anthesis. Embryos for enzymology studies were thus harvested between about 90 to 110 days postanthesis, a period when the wax synthase activity is high, lipid deposition has not reached maximum levels, and the seed coat is easily removed. The highest rate of increase of wax synthase activity is seen between days 80 and 90 postanthesis. Embryos for cDNA library construction were thus harvested between about 80 to 90 days postanthesis when presumably the rate of synthase of wax synthase protein would be maximal. Correspondingly, the level of mRNA encoding wax synthase would be presumed to be maximal at this stage.

B. Substrate Specificity

Acyl-CoA and alcohol substrates having varying carbon chain lengths and degrees of unsaturation were added to a microsomal membrane fraction having wax synthase activity to determine the range of substrates recognized by the jojoba wax synthase. Wax synthase activity was measured as described in Example 1, with acyl specificity measured using 80μM of acyl-CoA substrate and 100μM of radiolabeled

oleyl alcohol. Alcohol specificity was measured using 100μM of alcohol substrate and 40μM of radiolabeled eicosenoyl-CoA. Results of these experiments are presented in Table 1 below.

Table 1

Acyl and Alcohol Substrate Specificity of Jojoba Wax Synthase

Substrate	Wax synthase Activity (pmoles/min)	
Structure	Acyl Group	Alcohol Group
12:0	12	100
14:0	95	145
16:0	81	107
18:0	51	56
20:0	49	21
22:0	46	17
18:1	22	110
18:2	7	123
20:1	122	72
22:1	39	41
24:1	35	24

The above results demonstrate that the jojoba wax synthase utilizes a broad range of fatty acyl-CoA and fatty alcohol substrates.

In addition, wax synthase activity towards various acyl-thioester substrates was similarly tested using palmitoyl-CoA, palmitoyl-ACP and N-acetyl-S-palmitoyl cysteamine as acyl substrates. The greatest activity was observed with the acyl-CoA substrate. Significant activity (~10% of that with acyl-CoA) was observed with acyl-ACP, but no activity was detectable with the N-acetyl-S-palmitoyl cysteamine substrate.

C. Effectors of Activity

Various sulphydryl agents were screened for their effect on wax synthase activity. Organomercurial compounds were shown to strongly inhibit activity. Iodoacetamide and N-ethylmaleamide were much less effective. Inhibition by para-hydroxymercuribenzoate was observed, but this inhibition could be reversed by subsequent addition of DTT.

These results demonstrate that inhibition by para-hydroxymercuribenzoate involves blocking of an essential sulphydryl group.

D. Size Exclusion Chromatography

A column (1.5cm x 46cm) is packed with Sephacryl-200 (Pharmacia), sizing range: 5,000 - 250,000 daltons) and equilibrated with column buffer (25mM HEPES, 20% glycerol, 0.75% CHAPS, 1mM EDTA) containing 0.5M NaCl. Approximately 2 ml of a pooled concentrate from a single 1.5 M NaCl elution from a Blue A column (see Ex. 4C) is loaded and the column run at 0.5 ml/min. The eluted fractions are assayed for wax synthase activity according to the reconstitution protocol described in Example 1. Wax synthase activity appears as a broad peak beginning at the void fraction and decreasing throughout the remainder of the run. A portion of the fractions having wax synthase activity are treated with 1-^{14}C 16:0-CoA (0.0178 uM) for 15 minutes at room temperature. SDS is added to 2% and the samples are loaded on an SDS-PAGE gel. Following electrophoresis, the gel is blotted to Problott (Applied Biosystems; Foster City, CA) and the dried blot membrane analyzed by autoradiography. Alternatively, the blot may be scanned for radioactivity using an automated scanning system (AMBIS; San Diego, Ca.). In this manner, it is observed that the 57kD radiolabeled band tracks with wax synthase activity in the analyzed fractions.

Protein associated with wax synthase activity is further characterized by chromatography on a second size exclusion matrix. A fraction (100ul) of a 10X concentrated 1.5M NaCl elution from a Blue A column (following a 1.0M NaCl elution step) which contains wax synthase activity is chromatographed on a Superose 12 HR10/30 column (Pharmacia; Piscataway, NJ) and analyzed by Fast Protein Liquid Chromatography (FPLC) on a column calibrated with molecular weight standards (MW GF-70 and MW GF-1000; Sigma). Activity assays are performed on the eluted fractions. Most 53% of the recovered wax synthase activity is found in the void fractions, but an easily detectable activity is

found to elute at ~55kd according to the calibration curve. These data indicate the minimum size of an active native wax synthase protein is very similar to the 57kD size of the labeled band, thus providing evidence that wax synthase activity is provided by a single polypeptide. The fraction of wax synthase activity observed in the void fractions is presumably an aggregated form of the enzyme.

E. Palmitoyl-CoA Agarose Chromatography

A column (1.0 x 3cm) is packed with 16:0-CoA agarose (Sigma P-5297) and equilibrated with column buffer (See, Example 1, D.) containing 0.2M NaCl. Approximately 4 ml of a pooled concentrate from the 1.5M NaCl wash of the Blue A column is thawed and the salt concentration reduced by passage of the concentrate over a PD-10 (Pharmacia) desalting column equilibrated in 0.2M NaCl column buffer. The reduced salt sample (5ml) is loaded onto the 16:0 CoA agarose column at a flow rate of 0.15 ml/min. The column is washed with 0.5M NaCl column buffer and then with 1.5M NaCl column buffer. Although some wax synthase activity flows through the column or is removed by the 0.5M NaCl wash, the majority of the recovered activity (21% of the loaded activity) is recovered in the 1.5M NaCl eluted peak.

Portions of the fractions which demonstrate wax synthase activity are radiolabeled with [^{14}C]palmitoyl-CoA as described in Example 2 and analyzed by SDS polyacryamide gel electrophoresis (Laemmli, *Nature* (1970) *227*:680-685). Again the approximate 57kD radio labelled protein band is observed to track with wax synthase activity.

Example 4 - Purification of Jojoba Wax Synthase

Methods are described which may be used for isolation of a jojoba membrane preparation having wax synthase activity, solubilization of wax synthase activity and further purification of the wax synthase protein.

A. Microsomal Membrane Preparation

Jojoba embryos are harvested at approximately 90-110 days after flowering, as estimated by measuring water

content of the embryos (45-70%). The outer shells and seed coats are removed and the cotyledons quickly frozen in liquid nitrogen and stored at -70°C for future use. For initial protein preparation, frozen embryos are powdered by pounding in a steel mortar and pestle at liquid nitrogen temperature. In a typical experiment, 70g of embryos are processed.

The powder is added, at a ratio of 280ml of solution per 70g of embryos, to the following high salt solution: 3M NaCl, 0.3M sucrose, 100mM HEPES, 2mM DTT, and the protease inhibitors, 1mM EDTA, 0.7µg/ml leupeptin, 0.5µg/ml pepstatin and 17µg/ml PMSF. A cell free homogenate (CFH) is formed by dispersing the powdered embryos in the buffer with a tissue homogenizer (Kinematica, Switzerland; model PT10/35) for approximately 30 sec. and then filtering through three layers of Miracloth (CalBioChem, LaJolla, CA). The filtrate is centrifuged at 100,000 x g for one hour.

The resulting sample consists of a pellet, supernatant and a floating fat pad. The fat pad is removed and the supernatant fraction is collected and dialyzed overnight (with three changes of the buffering solution) versus a solution containing 1M NaCl, 100mM HEPES, 2mM DTT and 0.5M EDTA. The dialyzate is centrifuged at 200,000 x g for 1 1/2 hour to yield a pellet, DP2. The pellet is suspended in 25mM HEPES and 10% glycerol, at 1/20 of the original CFH volume, to yield the microsomal membrane preparation.

Activity is assayed as described in Example 1. Recovery of wax synthase activity is estimated at 34% of the original activity in the cell free homogenate. Wax synthase activity in this preparation is stable when stored at -70°C.

B. Solubilization of Wax synthase Protein

CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate) and NaCl are added to the microsomal membrane preparation to yield final concentrations of 2% and 0.5M, respectively. The samples are incubated on ice for approximately one hour and then diluted with 25mM

HEPES, 20% glycerol, 0.5M NaCl to lower the CHAPS concentration to 0.75%. The sample is then centrifuged at 200,000 x g for one hour and the supernatant recovered and assayed for wax synthase activity as described in Example 1.C. Typically, 11% of the wax synthase activity from the microsomal membrane preparation is recovered in the supernatant fraction. The solubilized wax synthase activity is stable when stored at -70°C.

C. Blue A Column Chromatography

A column (2.5 x 8cm) with a bed volume of approximately 30ml is prepared which contains Blue A (Cibacron Blue F3GA; Amicon Division, W.R. Grace & Co.), and the column is equilibrated with the column buffer (25mM HEPES, 20% glycerol, 0.75% CHAPS, 1mM EDTA) containing 0.4M NaCl. The solubilized wax synthase preparation is diluted to 0.4M NaCl by addition of column buffer (25mM HEPES, 20% glycerol, 0.75% CHAPS, 1mM EDTA) and loaded to the Blue A column.

The column is washed with column buffer containing 0.5M NaCl until no protein can be detected (as measured by absorbance at 280nm) in the buffer flowing through the column. Greater than 94% of the wax synthase activity binds to the column, while greater than 83% of other protein passes through. Typically, approximately 20% of the loaded wax synthase activity is recovered by elution. A portion of the recovered activity (17%) elutes with a 1.0M NaCl column buffer wash, while approximately 75% of the recovered activity elutes as a broad peak in a 150ml wash with 1.5M NaCl column buffer. Five ml fractions of the 1.5M wash are collected and assayed for wax synthase activity as described in Example 1. Fractions containing wax synthase activity are pooled and concentrated ten fold using an Amicon stirred cell unit and a YM30 membrane. The concentrated wax synthase preparation may be stored at -70°C.

D. SDS PAGE Analysis

Samples from the active BlueA column fractions are diluted in SDS PAGE sample buffer (1x buffer = 2% SDS, 30mM

DTT, 0.001% bromphenol blue) and analyzed by electrophoresis on 12% tris/glycine precast gels from NOVEX (San Diego, CA). Gels are run at 150V, constant voltage for approximately 1.5 hours. Protein is detected by silver staining (Blum *et al.*, *Electrophoresis* (1987) *8*:93-99). Careful examination of the gel reveals only a few polypeptides, including one of approximately 57kD, whose staining intensity in the various fractions can be correlated with the amount of wax synthase activity detected in those fractions. Furthermore, if radiolabeled [1-^{14}C]palmitoyl-CoA is added to the protein preparation prior to SDS PAGE analysis, autoradiography of the gel reveals that the 57kD labeled band tracks with wax synthase activity in these fractions. Other proteins are also present in the preparation, including the 56 and 54kD reductase proteins described in co-pending application USSN 07/767,251.

E. Continuous Phase Elution

Wax synthase protein is isolated for amino acid sequencing using an SDS-PAGE apparatus, Model 491 Prep Cell (Bio-Rad Laboratories, Inc., Richmond, CA), according to manufacturer's instructions. A portion (15 ml) of the wax synthase activity from the 1.5M NaCl elution of the Blue A column is concentrated 10 fold in a Centricon 30 (Amicon Division, W. R. Grace & Co.; Beverly, MA) and desalted with column buffer on a Pharmacia PD-10 desalting column. The sample is treated with 2% SDS and a small amount of bromphenol blue tracking dye and loaded onto a 5 ml, 4% acrylamide stacking gel over a 20 ml, 12% acrylamide running gel in the Prep Cell apparatus. The sample is electrophoresed at 10W and protein is continuously collected by the Prep Cell as it elutes from the gel. The eluted protein is then collected in 7.5-10 ml fractions by a fraction collector. One milliliter of each fraction in the area of interest (based on the estimated 57kD size of the wax synthase protein) is concentrated to 40 µl in a Centricon 30 and treated with 2% SDS. The samples are run on 12% acrylamide mini-gels (Novex) and stained with

silver. Various modifications to the continuous phase elution process in order to optimize for wax synthase recovery may be useful. Such modifications include adjustments of acrylamide percentages in gels volume of the gels, and adjustments to the amount of wax synthase applied to the gels. For example, to isolate greater amounts of the wax synthase protein the Blue A column fractions may be applied to larger volume, 20-55 ml, acrylamide gels at a concentration of approximately 1 mg of protein per 20 ml of gel. The protein fractions eluted from such gels may then be applied 10-15% gradient acrylamide gels for increased band separation.

The protein content of each fraction is evaluated visually and fractions containing wax synthase protein are pooled and concentrated for amino acid sequencing. In order to maximize the amount of wax synthase enzyme collected, fractions which also contain the 56kD reductase protein band are included in the pooled preparation. As the reductase protein sequence is known (see Figure 1), further purification of wax synthase protein in the pooled preparation is not necessary prior to application of amino acid sequencing techniques (see Example 5).

G. Blotting Proteins to Membranes

Alternatively, wax synthase protein may be further isolated for amino acid sequencing by transfer to PVDF membranes following SDS-PAGE, either Immobilon-P (Millipore; Bedford, MA) or ProBlott (Applied Biosystems; Foster City, CA). Although transfer to nitrocellulose may also be useful, initial studies indicate poor transfer to nitrocellulose membranes, most likely due to the hydrophobic nature of this protein. PVDF membranes, such as ProBlott and Immobilon-P find preferential use in different methods, depending on the amino acid sequencing technique to be employed. For example, transfer to ProBlott is useful for N-terminal sequencing methods and for generation of peptides from cyanogen bromide digestion, Immobilon-P is preferred.

1. *Blotting to Nitrocellulose*: When protein is electroblotted to nitrocellulose, the blotting time is typically 1-5 hours in a buffer such as 25mM Tris, 192mM glycine in 5-20% methanol. Following electroblotting, membranes are stained in 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid for 2 minutes and destained in 2-3 changes of 0.1% (v/v) acetic acid, 2 minutes for each change. These membranes are then stored wet in heat-sealed plastic bags at -20°C. If time permits, blots are not frozen but used immediately for digestion to create peptides for determination of amino acid sequence as described below.

2. *Blotting to PVDF*: When protein is electroblotted to Immobilon P PVDF, the blotting time is generally about 1-2 hours in a buffer such as 25mM Tris/192mM glycine in 20% (v/v) methanol. Following electroblotting to PVDF, membranes are stained in 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol/10% (v/v) acetic acid for 5 minutes and destained in 2-3 changes of 50% (v/v) methanol/10% (v/v) acetic acid, 2 minutes for each change. PVDF membranes are then allowed to air dry for 30 minutes and are then stored dry in heat-sealed plastic bags at -20°C. Protein blotted to PVDF membranes such as Pro Blott, may be used directly to determine N-terminal sequence of the intact protein. A protocol for electroblotting proteins to ProBlott is described below in Example 5A.

Example 5 - Determination of Amino Acid Sequence

In this example, methods for determination of amino acid sequences of plant proteins associated with wax synthase activity are described.

A. Cyanogen Bromide Cleavage of Protein and Separation of Peptides

Cyanogen bromide cleavage is performed on the protein of interest using the methodology described in the Probe-Design Peptide Separation System Technical Manual from Promega, Inc. (Madison, WI). The wax synthase protein, if not available in a purified liquid sample, is blotted to a

PVDF membrane as described above. Purified wax synthase protein or wax synthase bands from the PVDF blot, are placed in a solution of cyanogen bromide in 70% (v/v) formic acid, and incubated overnight at room temperature. Following this incubation the cyanogen bromide solutions are removed, pooled and dried under a continuous nitrogen stream using a Reacti-Vap Evaporator (Pierce, Rockford, IL). Additional elution of cyanogen bromide peptides from PVDF may be conducted to ensure complete removal, using a peptide elution solvent such as 70% (v/v) isopropanol, 0.2% (v/v) trifuoroacetic acid, 0.1mM lysine, and 0.1mM thioglycolic acid. The elution solvents are then removed and added to the tube containing the dried cyanogen bromide solution, and dried as described above. The elution procedure may be repeated with fresh elution solvent. 50µl of HPLC grade water is then added to the dried peptides and the water removed by evaporation in a Speed-Vac (Savant, Inc., Farmingdale, NY).

Peptides generated by cyanogen bromide cleavage are separated using a Tris/Tricine SDS-PAGE system similar to that described by Schägger and von Jagow (*Anal. Biochem.* (1987) *166*:368-379). Gels are run at a constant voltage of 125-150 volts for approximately 1 hour or until the tracking dye has begun to run off the bottom edge of the gel. Gels are soaked in transfer buffer (125mM Tris, 50mM glycine, 10% (v/v) methanol) for 15-30 minutes prior to transfer. Gels are blotted to ProBlott sequencing membranes (Applied Biosystems, Foster City, CA) for 2 hours at a constant voltage of 50 volts. The membranes are stained with Coomassie blue (0.1% in 50% (v/v) methanol/10% (v/v) acetic acid) and destained for 3X 2 min. in 50% (v/v) methanol/10% (v/v) acetic acid. Membranes are air-dried for 30-45 minutes before storing dry at -20° C.

Peptides blotted on to ProBlott can be directly loaded to the sequencer cartridge of the protein sequencer without the addition of a Polybrene-coated glass fibre filter. Peptides are sequenced using a slightly modified reaction cycle, BLOT-1, supplied by Applied Biosystems. Also,

solution S3 (butyl chloride), is replaced by a 50:50 mix of S1 and S2 (n-heptane and ethyl acetate). These two modifications are used whenever samples blotted to ProBlott are sequenced.

B. Protease Digestion and Separation of Peptides

Purified wax synthase protein provided in a liquid solution or wax synthase proteins blotted to nitrocellulose may be subjected to digestion with proteases in order to obtain peptides for sequencing. The method used is that of Aebersold, *et al.* (*PNAS* (1987) *84*:6970).

For protein provided on nitrocellulose, bands of the wax synthase proteins, and also an equal amount of blank nitrocellulose to be used as a control, are cut out of the nitrocellulose membrane and washed several times with HPLC grade water in order to remove the Ponceau S. Following this wash, 1.0ml of 0.5% polyvinylpyrrolidone (PVP-40, Aldrich, Milwaukee, WI) in 0.5% acetic acid is added to the membrane pieces and this mixture is incubated for 30 minutes at 37°C. In order to remove the PVP-40 completely, nitrocellulose pieces are washed with many volumes of HPLC grade water (8 x 5ml), checking the absorbance of the washes at 214nm on a spectrophotometer. Also, PVP-40 is more easily removed if bands are not cut into small pieces until after PVP-40 treatment and washing.

The proteins, in solution or on nitrocellulose pieces, are then suspended in an appropriate digest buffer, for example trypsin digest buffer, 100mM sodium bicarbonate pH 8.2, or endoproteinase gluC buffer, 25mM ammonium carbonate/1mM EDTA, pH 7.8. Acetonitrile is added to the digest mixture to a concentration of 5-10% (v/v). Proteases are diluted in digest buffer and added to the digest mixture, typically at a ratio of 1:10 (w/w) protease to protein. Digests are incubated 18-24 hours. For example, trypsin digests are incubated at 37°C and endoproteinase gluC digests are incubated at room temperature. Similarly, other proteases may be used to digest the wax synthase proteins, including lysC and aspN. While the individual digest buffer conditions may be

different, the protocols for digestion, peptide separation, purification and sequencing are substantially the same as those described for digestion with trypsin and gluC.

Following overnight incubation, digest reactions are stopped by the addition of 10µl 10% (v/v) trifluoroacetic acid (TFA) or 1µl 100% TFA. When the protein is provided on nitrocellulose, the nitrocellulose pieces are washed with 1-5 100µl volumes of digest buffer with 5-10% acetonitrile, and these volumes are concentrated to a volume of less than 100µl in a Speed-Vac.

The peptides resulting from digestion are separated on a Vydac reverse phase C18 column (2.1mm x 100mm) installed in an Applied Biosystems (Foster City, CA) Model 130 High Performance Liquid Chromatograph (HPLC). Mobile phases used to elute peptides are: Buffer A: 0.1mM sodium phosphate, pH2.2; Buffer B: 70% acetonitrile in 0.1mM sodium phosphate, pH2.2. A 3-step gradient of 10-55% buffer B over two hours, 55-75% buffer B over 5 minutes, and 75% buffer B isocratic for 15 minutes at a flow rate of 50µl/minute is used. Peptides are detected at 214nm, collected by hand, and then stored at -20° C.

Due to the hydrophobic nature of the wax synthase proteins, addition of a detergent in enzyme digestions buffers may be useful. For example, fractions from the continuous phase elution procedure described above which contain the jojoba wax synthase are concentrated in a Centricon 30 in 100mM $NaHCO_3$/1.0% CHAPS to a final volume of 110µl. Two µg of trypsin in 5µl of 100mM Na HCO_3/1.0% CHAPS is added to the protein solution and the mixture is incubated overnight at 37°C, and the digestion stopped by addition of trifluoroacetic acid (TFA). The sample is centrifuged lightly and the peptides separated on a Vydac C18 column and eluted as described above. In this procedure, the CHAPS elutes at ~40-53% Buffer B, and obscures the peptide peaks in this region.

Where the primary separation yields a complex peptide pattern, such as where excess protein is used or contaminants (such as the jojoba reductase protein) are

present, peptide peaks may be further chromatographed using the same column, but a different gradient system. For the above jojoba wax synthase preparation, hydrophilic peaks were separated using a gradient of 0-40% Buffer B for 60 minutes, 40-75% B for 35 minutes and 75-100% B for 10 minutes. Hydrophobic peaks were separated using 0-40% Buffer B for 40 minutes, 40-80% B for 60 minutes and 80-100% B for 10 minutes. For these separations, Buffer A is 0.1% TFA and Buffer B is 0.1% TFA in acetonitrile.

C. N-terminal Sequencing of Proteins and Peptides

All sequencing is performed by Edman degradation on an Applied Biosystems 477A Pulsed-Liquid Phase Protein Sequencer; phenylthiohydantoin (PTH) amino acids produced by the sequencer are analyzed by an on-line Applied Biosystems 120A PTH Analyzer. Data are collected and stored using an Applied BioSystems model 610A data analysis system for the Apple Macintosh and also on to a Digital Microvax using ACCESS*CHROM software from PE NELSON, Inc. (Cupertino, CA). Sequence data is read from a chart recorder, which receives input from the PTH Analyzer, and is confirmed using quantitative data obtained from the model 610A software. All sequence data is read independently by two operators with the aid of the data analysis system.

For peptide samples obtained as peaks off of an HPLC, the sample is loaded on to a Polybrene coated glass fiber filter (Applied Biosystems, Foster City, CA) which has been subjected to 3 pre-cycles in the sequencer. For peptides which have been reduced and alkylated, a portion of the PTH-amino acid product material from each sequencer cycle is counted in a liquid scintillation counter. For protein samples which have been electroblotted to Immobilon-P, the band of interest is cut out and then placed above a Polybrene coated glass fiber filter, pre-cycled as above and the reaction cartridge is assembled according to manufacturer's specifications. For protein samples which have been electroblotted to ProBlott, the glass fiber filter is not required.

In order to obtain protein sequences from small amounts of sample (5-30 pmoles), the 477A conversion cycle and the 120A analyzer as described by Tempst and Riviere (*Anal. Biochem.* (1989) *183*:290).

Amino acid seqeunce of jojoba wax synthase peptides obtained by trypsin digestion as described above are presented in Table 2 below.

Table 2

Amino Acid Sequence of Jojoba Wax Synthase Tryptic Peptides

SQ1114	ETYVPESVTKK
SQ1084	VPXEPSIAAX
SQ1083	ETYVPEEvtk
SQ1120	DLMAVAGEAlk
SQ1125	MTNVKPYIPDF
SQ1129	FLPXXVAiTGe
SQ1131	FGNTSSXXLyxelayak
SQ1137	AEAEEVMYGAIDEVLEK

The amino acid sequence of wax synthase peptides is respresented using the one letter code. "X" represents a position where the amino acid could not be identified, and amino acids represented by lower case letters represent residues which were identified with a lesser degree of confidence.

Example 6 - Purification of Additional Wax Synthases

Adaptation of jojoba wax synthase solubilization and purification methods to obtain partially purified preparations of wax synthase from other organisms are described.

A. Acinetobacter

Cells of *Acinetobacter calcoaceticus* strain BD413 (ATCC #33305) are grown on ECLB (*E. coli* luria browth), collected during the logarithmic growth phase and washed in a buffer containing; Hepes, pH 7.5, 0.1M NaCl, 1mM DTT and

protease inhibitors. Washed cells were resuspended in fresh buffer and ruptured by passage through a French pressure cell (two passes at ~16,000p.s.i.). Unbroken cells are removed by centrifugation at 5000 x g for 10 minutes, and membranes are collected by centrifugation at 100,000 x g for 1 hour. The membrane pellet is homogenized in storage buffer (25mM Hepes, pH 7.5, 10% (w/v) glycerol). Wax synthase activity is detected in these membranes using assay conditions described for the jojoba enzyme in Example 1B, using [1-^{14}C] palmitoyl-CoA and 18:1 alcohol as the substrates.

Wax synthase activity is solubilized by incubation of the membranes with 2% CHAPS in the presence of 0.5M NaCl, as described for the jojoba enzyme in Example 4B. Solubilization of the activity is demonstrated by the detection of wax synthase enzyme activity in the supernatant fraction after centrifugation at 200,000g for 1 hour and by size exclusion chromatography (i.e. the activity elutes from the column in the retained fractions as a symmetrical peak). The activity of the solubilized enzyme is detected by simple dilution of the CHAPS concentration to ~0.3% (i.e. to below its CMC). Incorporation of the enzyme into phospholipid vesicles is not required to detect solubilized activity.

For purification, the solubilized *Acinetobacter* wax synthase activity is subjected to chromatographic purification procedures similar to those described for the jojoba acyl-CoA reductase. The soluble protein preparation is loaded to a Blue A agarose column under low salt conditions (150mM NaCl in a column buffer containing 0.75% CHAPS, 10% glycerol, 25mM Hepes, pH 7.5) and eluted from the column using 1.0M NaCl in the column buffer.

Size exclusion chromatography on Superose 12 (Pharmacia; Piscataway, NJ) medium is used to obtain an estimate of the size of the native enzyme and to aid in identifying candidate polypeptides. Comparison to molecular mass standards chromatographed under identical conditions yields an estimate of ~46kD for the native wax

synthase activity. Three polypeptides bands, with apparent molecular masses of 45kD, 58kD and 64kD, were identified which tracked with wax synthase activity. N-terminal sequence of the 45kD polypeptide, the strongest candidate for wax synthase, is determined as XDIAIIGSGsAGLAQaxilkdag, where the one letter code for amino acids is used, "X" represents a position where the amino acid could not be identified, and amino acids represented by lower case letters represent residues which were identified with a lesser degree of confidence.

B. Euglena

Euglena gracilis, strain Z (ATCC No. 12716) is grown heterotrophically in the dark (Tani *et al.* (1987) *Agric. Biol. Chem.* *51*:225-230) at ~26°C with moderate shaking. Cells are collected and washed in buffer containing 25mM Bis-Tris-Propane, pH 7.0, 0.25M NaCl and 1mM EDTA. Washed cells are resuspended in fresh buffer and ruptured by passage through a French pressure cell (two passes at ~16,000 p.s.i.). Unbroken cells, cell debris and nuclei are removed by centrifugation at 20,000 x g for 20 minutes, and microsomal membranes are collected by centrifugation at 200,000 x g for 1 hour. The membrane pellet is homogenized in storage buffer (25mM Bis-Tris-Propane, pH 7.0, 0.25M NaCl, 10% (w/v) glycerol and 1mM EDTA). Wax synthase activity is detected in these membranes using assay conditions as described for the jojoba enzyme. The radiolabelled substrate is the same as for the jojoba example (i.e. [1-^{14}C] palmitoyl-CoA), however, 16:0 rather than 18:1 is used as the alcohol acceptor, and Bis-Tris-Propane buffer at pH 7.0 is utilized.

The *Euglena* wax synthase activity is solubilized by incubation of the membranes with 2% CHAPS in the presence of 0.5M NaCl. Solubilization of the protein is demonstrated by the detection of enzyme activity in the supernatant fraction after centrifugation at 200,000 x g for 1 hour. The activity of the solubilized enzyme is detected by dilution of the CHAPS concentration to ~0.3% (i.e. to below its CMC). It is not necessary to

incorporate the enzyme into phospholipid vesicles as was the case for the solubilized jojoba wax synthase.

For partial purification, the solubilized *Euglena* wax synthase activity is subjected to chromatographic separation on Blue A agarose medium. The column is equilibrated with 0.1M NaCl in a column buffer containing; 25mM Bis-Tris-Propane, pH 7.0, 20% (w/v) glycerol, 0.75% CHAPS and 1mM EDTA. The sample containing solubilized wax synthase activity is diluted to 0.1M NaCl and loaded onto a 1 x 7cm column (5.5ml bed volume). The column is washed with equilibration buffer and subjected to a linear NaCl gradient (0.1M to 1.0M NaCl) in column buffer. Wax synthase activity is eluted as a broad peak in the last half of the salt gradient.

SDS-PAGE analysis of column fractions reveals that the polypeptide complexity of the activity eluted from the column is greatly reduced relative to the loaded material. A polypeptide with an apparent molecular mass of ~41kD was observed to track with wax synthase activity in the column fractions. Further purification techniques, such as described for jojoba and *Acinetobacter* are conducted to verify the association of wax synthase activity with the ~41kD peptide.

For further analysis of wax synthase activity in *Euglena*, size exclusion chromatography was conducted as follows. A microsomal membrane preparation was obtained from *Euglena* cells grown on liquid, heterotrophic, medium (Tani *et al., supra*) in the dark. Wax synthase activity was solubilized by treating the membranes with 2% (w/v) CHAPS and 500mM NaCl in a buffered solution (25mM Bis-Tris, pH 7.0, 1mM EDTA and 10% (w/v) glycerol) for 1 hour on ice. After dilution of the CHAPS to 0.75% and the NaCl to 200mM by addition of a dilution buffer, the sample was centrifuged at ~200,000 x g for 1.5 hours. The supernatant fraction was loaded onto a Blue A dye column pre-equilibrated with Column Buffer (25mM Bis-Tris pH 7.0, 1mM EDTA, 10% glycerol, 0.75% CHAPS) which also contained 200mM NaCl. The column was washed with Column Buffer containing

200mM NaCl until the A280 of the effluent returned to the preload value. Wax synthase activity which had bound to the column was released by increasing the NaCl concentration in the Column Buffer to 1.5M. The fractions from the Blue A column containing wax synthase activity released by the 1.5M NaCl (~20ml combined volume) were pooled and concentrated approximately 30-fold via ultrafiltration (Amicon pressure cell fitted with a YM 30 membrane). The concentrated material from the Blue A column was used as the sample for a separation via size exclusion chromatography on Superose 12 medium (Pharmacia).

Approximately 200µl of the sample was loaded onto a Superose 12 column (HR 10/30), pre-equilibrated with Column Buffer containing 0.5M NaCl, and developed at a flow rate of 0.1ml/min. The wax synthase activity eluted from the column as a smooth peak. Comparison of the elution volume of the wax synthase activity with the elution profiles of molecular mass standard proteins yielded an estimate of 166kD for the apparent molecular mass of the enzyme. Fractions which contained wax synthase activity were analyzed via SDS-polyacrylamide gel electrophoresis followed by silver staining. A preliminary analysis of the polypeptide profiles of the various fractions did not reveal any proteins with molecular masses of 100kD or greater whose staining intensity appeared to match the activity profile. The wax synthase polypeptide may be present as a minor component in the sample mixture that is not readily detectable on the silver-stained gel. Alternatively, the enzyme may be composed of subunits which are dissociated during SDS-PAGE.

Example 7 - Isolation of Wax Synthase Nucleic Acid Sequences

Isolation of wax synthase nucleic acid sequences from jojoba embryo cDNA libraries or from genomic DNA is described.

A. Construction of Jojoba cDNA Libraries

RNA is isolated from jojoba embryos collected at 80-90 days post-anthesis using a polyribosome isolation method, initially described by Jackson and Larkins (*Plant Physiol.* (1976) *57*:5-10), as modified by Goldberg *et al.* (*Developmental Biol.* (1981) *83*:201-217). In this procedure all steps, unless specifically stated, are carried out at 4°C. 10gm of tissue are ground in liquid nitrogen in a Waring blender until the tissue becomes a fine powder. After the liquid nitrogen has evaporated, 170ml of extraction buffer (200mM Tris pH 9.0, 160mM KC1, 25mM EGTA, 70mM MgC12, 1% Triton X-100, 05% sodium deoxycholate, 1mM spermidine, 10mM ß-mercaptoethanol, and 500mM sucrose) is added and the tissue is homogenized for about 2 minutes. The homogenate is filtered through sterile miracloth and centrifuged at 12,000 x g for 20 minutes. The supernatant is decanted into a 500ml sterile flask, and 1/19 volume of a 20% detergent solution (20% Brij 35, 20% Tween 40, 20% Noidet p-40 w/v) is added at room temperature. The solution is stirred at 4°C for 30 minutes at a moderate speed and the supernatant is then centrifuged at 12,000 x g for 30 minutes.

About 30ml of supernatant is aliquoted into sterile Ti 60 centrifuge tubes and underlaid with 7ml of a solution containing 40mM Tris pH 9.0, 5mM EGTA, 200mM KC1, 30mM MgC12, 1.8M sucrose, 5mM ß-mercaptoethanol. The tubes are filled to the top with extraction buffer, and spun at 60,000 rpm for 4 hours at 4°C in a Ti60 rotor. Following centrifugation, the supernatant is aspirated off and 0.5ml of resuspension buffer (40mM Tris pH 9.0, 5mM EGTA, 200mM KC1, 30mM $MgCl_2$, 5mM ß-mercaptoethanol) is added to each tube. The tubes are placed on ice for 10 minutes, after which the pellets are thoroughly resuspended and pooled. The supernatant is then centrifuged at 120 x g for 10 minutes to remove insoluble material. One volume of self-digested 1mg/ml proteinase K in 20mM Tris pH 7.6, 200mM EDTA, 2% N-lauryl-sarcosinate is added to the supernatant

and the mixture incubated at room temperature for 30 minutes.

RNA is precipitated by adding 1/10 volume of sodium acetate and 2 volumes of ethanol. After several hours at -20°C RNA is pelleted by centrifugation at 12,000 x g at 4°C for 30 minutes. The pellet is resuspended in 10ml of TE buffer (10mM Tris, 1mM EDTA) and extracted with an equal volume of Tris pH 7.5 saturated phenol. The phases are separated by centrifuging at 10,000 x g for 20 minutes at 4°C. The aqueous phase is removed and the organic phase is re-extracted with one volume of TE buffer. The aqueous phases are then pooled and extracted with one volume of chloroform. The phases are again separated by centrifugation and the aqueous phase ethanol precipitated as previously described, to yield the polyribosomal RNA.

Polysaccharide contaminants in the polyribosomal RNA preparation are removed by running the RNA over a cellulose column (Sigma-cell 50) in high salt buffer (0.5M NaCl, 20mM Tris pH 7.5, 1mM EDTA, 0.1% SDS). The contaminant binds to the column and the RNA is collected in the eluant. The eluant fractions are pooled and the RNA is ethanol precipitated. The precipitated total RNA is then resuspended in a smaller volume and applied to an oligo d(T) cellulose column to isolate the polyadenylated RNA.

Polyadenylated RNA is used to construct a cDNA library in the plasmid cloning vector pCGN1703, derived from the commercial cloning vector Bluescribe M13- (Stratagene Cloning Systems; San Diego, CA), and made as follows. The polylinker of Bluescribe M13- is altered by digestion with *Bam*HI, treatment with mung bean endonuclease, and blunt-end ligation to create a *Bam*HI-deleted plasmid, pCGN1700. pCGN1700 is digested with *Eco*RI and *Sst*I (adjacent restriction sites) and annealed with a synthetic linker having restriction sites for *Bam*HI, *Pst*I, *Xba*I, *Apa*I and *Sma*I, a 5' overhang of AATT, and a 3' overhang of TCGA. The insertion of the linker into pCGN1700 eliminates the *Eco*RI site, recreates the *Sst*I (also, sometimes referred to as "*Sac*I" herein) site found in Bluescribe, and adds the

new restriction sites contained on the linker. The resulting plasmid pCGN1702, is digested with *Hin*dIII and blunt-ended with Klenow enzyme; the linear DNA is partially digested with *Pvu*II and ligated with T4 DNA wax synthase in dilute solution. A transformant having the *lac* promoter region deleted is selected (pCGN1703) and is used as the plasmid cloning vector.

Briefly, the cloning method for cDNA synthesis is as follows. The plasmid cloning vector is digested with *Sst*I and homopolymer T-tails are generated on the resulting 3'-overhang stick-ends using terminal deoxynucleotidyl transferase. The tailed plasmid is separated from undigested or un-tailed plasmid by oligo(dA)-cellulose chromatography. The resultant vector serves as the primer for synthesis of cDNA first strands covalently attached to either end of the vector plasmid. The cDNA-mRNA-vector complexes are treated with terminal transferase in the presence of deoxyguanosine triphosphate, generating G-tails at the ends of the cDNA strands. The extra cDNA-mRNA complex, adjacent to the *Bam*HI site, is removed by *Bam*HI digestion, leaving a cDNA-mRNA-vector complex with a *Bam*HI stick-end at one end and a G-tail at the other. This complex is cyclized using an annealed synthetic cyclizing linker which has a 5' *Bam*HI sticky-end, recognition sequences for restriction enzymes *Not*I, *Eco*RI and *Sst*I, and a 3' C-tail end. Following ligation and repair the circular complexes are transformed into *E. coli* strain DH5α (BRL, Gaithersburg, MD) to generate the cDNA library. The jojoba embryo cDNA bank contains between approximately 1.5×10^6 clones with an average cDNA insert size of approximately 500 base pairs.

Additionally, jojoba polyadenylated RNA is also used to construct a cDNA library in the cloning vector λZAPII/*Eco*RI (Stratagene, San Diego, CA). The library is constructed using protocols, DNA and bacterial strains as supplied by the manufacturer. Clones are packaged using Gigapack Gold packaging extracts (Stratagene), also according to manufacturer's recommendations. The cDNA

library constructed in this manner contains approximately 1 x 10^6 clones with an average cDNA insert size of approximately 400 base pairs.

B. Polymerase Chain Reaction

Using amino acid sequence information obtained as described in Example 5, nucleic acid sequences of wax synthase proteins are obtained by polymerase chain reaction (PCR). Synthetic oligonucleotides are synthesized which correspond to the amino acid sequence of selected wax synthase peptide fragments. If the order of the fragments in the wax synthase protein is known, such as when one of the peptides is from the N-terminus or the selected peptides are contained on one long peptide fragment, only one oligonucleotide primer is needed for each selected peptide. The oligonucleotide primer for the more N-terminal peptide, forward primer, contains the encoding sequence for the peptide. The oligonucleotide primer for the more C-terminal peptide, reverse primer, is complementary to the encoding sequence for the selected peptide. Alternatively, when the order of the selected peptides is not known, two oligonucleotide primers are required for each peptide, one encoding the selected amino acid sequence and one complementary to the selected amino acid sequence. Any sequenced wax synthase peptides may be selected for construction of oligonucleotides, although more desirable peptides are those which contain amino acids which are encoded by the least number of codons, such as methionine, tryptophan, cysteine, and other amino acids encoded by fewer than four codons. Thus, when the oligonucleotides are mixtures of all possible sequences for a selected peptide, the number of degenerate oligonucleotides may be low.

PCR is conducted with these oligonucleotide primers using techniques that are well known to those skilled in the art. Jojoba nucleic acid sequences, such as reverse transcribed cDNA, DNA isolated from the cDNA libraries described above or genomic DNA, are used as template in these reactions. In this manner, segments of wax synthase

DNA are produced. The PCR products are analyzed by gel electrophoresis techniques to select those reactions yielding a desirable wax synthase fragment.

C. Screening Libraries for Wax Synthase Sequences

Wax synthase DNA fragments obtained by PCR are labeled and used as a probe to screen clones from the cDNA libraries described above. DNA library screening techniques are known to those in the art and described, for example in Maniatis *et al.* (*Molecular Cloning: A Laboratory Manual, Second Edition* (1989) Cold Spring Harbor Laboratory Press). In this manner, wax synthase nucleic acid sequences are obtained which may be analyzed for nucleic acid sequence and used for expression of wax synthase in various hosts, both procaryotic and eucaryotic.

An approximately 1500 nucleotide cDNA clone is obtained in this manner. Comparison to the wax synthase peptide fragments provided in Table 2 reveals the presence of each of these peptides in the translated sequence, with the exception of SQ1129. Northern analysis of jojoba embryo RNA indicates that the wax synthase mRNA is approximately 2kb in length. Additional wax synthase nucleic acid sequence is obtained using further PCR techniques, such as 5' RACE (Frohman *et al.*, *Proc. Nat. Acad. Sci.* (1988) *85*:8998-9002). Alternatively, additional sequences may be obtained by rescreening cDNA libraries or from genomic DNA. DNA sequence of a jojoba wax synthase gene is presented in Figure 2. A plasmid containging the entire wax synthase sequence in pCGN1703 is designated pCGN7614.

D. Expression of Wax Synthase in E. coli

The wax synthase gene from pCGN7614 is placed under the control of the Tac promoter of *E. coli* expression vector pDR540 (Pharmacia) as follows. pCGN7614 DNA is digested at the vector *Sal*I sites and the ends are partially filled in using the Klenow fragment of DNA polymerase I and the nucleotides TTP and dCTP. The pDR540 vector is prepared by digesting with *Bam*HI and partially filling in the ends with dGTP and dATP. The 1.8 kb

fragment from pCGN7614 and the digested pDR540 vector are gel purified using low melting temperature agarose and ligated together using T4 DNA ligase. A colony containing the wax synthase in the sense orientation relative to the *E. coli* promoter was designated pCGN7620, and a colony containing the wax synthase gene in the antisense orientation was designated pCGN7621. To assay for wax synthase activity, 50 ml cultures of pCGN7620 and pCGN7621 are grown to log phase in liquid culture, and induced for 2 hours by the addition of IPTG to a concentration of 1mM. The cells are harvested by centrifugation and subjected to the assay for wax synthase activity as described for jojoba extracts. TLC analysis indicates that the cell extract from pCGN7620 directs synthesis of wax ester, while the control extract from pCGN7621 does not direct the synthesis of wax ester.

Example 8 - Wax Synthase and Reductase Constructs for Plant Expression

Constructs which provide for expression of wax synthase and reductase sequences in plant cells may be prepared as follows.

A. Expression Cassettes

Expression cassettes which contain 5' and 3' regulatory regions from genes expressed preferentially in seed tissues may be prepared from napin, Bce4 and ACP genes as described, for example in WO 92/03564.

For example, napin expression cassettes may be prepared as follows. A napin expression cassette, pCGN1808, which may be used for expression of wax synthase or reductase gene constructs is described in Kridl *et al.* (*Seed Science Research* (1991) *1*:209-219), which is incorporated herein by reference.

Alternatively, pCGN1808 may be modified to contain flanking restriction sites to allow movement of only the expression sequences and not the antibiotic resistance marker to binary vectors such as pCGN1557 (McBride and Summerfelt, *supra*). Synthetic oligonucleotides containing

*Kpn*I, *Not*I and *Hin*dIII restriction sites are annealed and ligated at the unique *Hin*dIII site of pCGN1808, such that only one *Hin*dIII site is recovered. The resulting plasmid, pCGN3200 contains unique *Hin*dIII, *Not*I and *Kpn*I restriction sites at the 3'-end of the napin 3'-regulatory sequences as confirmed by sequence analysis.

The majority of the napin expression cassette is subcloned from pCGN3200 by digestion with *Hin*dIII and *Sac*I and ligation to *Hin*dIII and *Sac*I digested pIC19R (Marsh, *et al.* (1984) *Gene 32*:481-485) to make pCGN3212. The extreme 5'-sequences of the napin promoter region are reconstructed by PCR using pCGN3200 as a template and two primers flanking the *Sac*I site and the junction of the napin 5'-promoter and the pUC backbone of pCGN3200 from the pCGN1808 construct. The forward primer contains *Cla*I, *Hin*dIII, *Not*I, and *Kpn*I restiction sites as well as nucleotides 408-423 of the napin 5'-sequence (from the *Eco*RV site) and the reverse primer contains the complement to napin sequences 718-739 which include the unique *Sac*I site in the 5'-promoter. The PCR was performed using a Perkin Elmer/Cetus thermocycler according to manufacturer's specifications. The PCR fragment is subcloned as a blunt-ended fragment into pUC8 (Vieira and Messing (1982) *Gene 19*:259-268) and digested with *Hin*cII to give pCGN3217. Sequence of pCGN3217 across the napin insert verifies that no improper nucleotides were introduced by PCR. The napin 5-sequences in pCGN3217 are ligated to the remainder of the napin expression cassette by digestion with *Cla*I and *Sac*I and ligation to pCGN3212 digested with *Cla*I and *Sac*I. The resulting expression cassette pCGN3221, is digested with *Hin*dIII and the napin expression sequences are gel purified away and ligated to pIC20H (Marsh, *supra*) digested with *Hin*dIII. The final expression cassette is pCGN3223, which contains in an ampicillin resistant background, essentially identical 1.725 napin 5' and 1.265 3' regulatory sequences as found in pCGN1808. The regulatory regions are flanked with *Hin*dIII, *Not*I and *Kpn*I restriction sites and unique

*Sal*I, *Bgl*II, *Pst*I, and *Xho*I cloning sites are located between the 5' and 3' noncoding regions.

Wax synthase gene sequences are inserted into such cassettes to provide expression constructs for plant transformation methods. For example, such constructs may be inserted into binary vectors for *Agrobacterium*-mediated transformation as described below.

B. Wax Synthase Constructs for Plant Transformation

The wax synthase gene plasmid pCGN7614 is digested with *Afl*III, and ligated with adapters to add *Bcl*I sites to the *Afl*III sticky ends, followed by digestion with *Sal*I and *Bcl*I. The fragment containing the wax synthase gene is gel purified and cloned into *Sal*I/*Bam*HI digested pCGN3223, a napin expression cassette. The resulting plasmid which contains the wax synthase gene in a sense orientation in the napin expression cassette is designated pCGN7624. DNA isolated from pCGN7624 is digested with *Asp*718 (a *Kpn*I isoschizimer), and the napin/wax synthase fusion gene is cloned into *Asp*718 digested binary vector pCGN1578 (McBride and Summerfelt, *supra*). The resultant binary vector, designated pCGN7626, is transformed into *Agrobacterium* strain EHA101 and used for transformation of *Arabidopsis* and rapeseed explants.

C. Reductase Constructs for Plant Transformation

Constructs for expression of reductase in plant cells using 5' and 3' regulatory regions from a napin gene, are prepared.

A reductase cDNA (in the pCGN1703 vector described above) designated pCGN7571, is digested with *Sph*I (site in 3' untranslated sequence at bases 1594-1599) and a *Sal*I linker is inserted at this site. The resulting plasmid is digested with *Bam*HI and *Sal*I and the fragment containing the reductase cDNA gel purified and cloned into *Bgl*II/*Xho*I digested pCGN3223, the napin cassette described above, resulting in pCGN7585.

A *Hind*III fragment of pCGN7585 containing the napin 5'/reductase/napin 3' construct is cloned into HindIII

digested pCGN1578 (McBride and Summerfelt, *supra*), resulting in pCGN7586, a binary vector for plant transformation.

Plant transformation construct pCGN7589, also containing the jojoba reductase gene under expression of a napin promoter, is prepared as follows. pCGN7571 is in vitro mutagenized to introduce an *Nde*I site at the first ATG of the reductase coding sequence and a *Bgl*II site immediately upstream of the *Nde*I site. *Bam*HI linkers are introduced into the *Sph*I site downstream of the reductase coding region. The 1.5 kb *Bgl*II-*Bam*HI fragment is gel purified and cloned into *Bgl*II-*Bam*HI digested pCGN3686 (see below), resulting in pCGN7582.

pCGN3686 is a cloning vector derived from Bluescript KS+ (Stratagene Cloning Systems; San Diego, CA), but having a chloramphenicol resistance gene and a modified linker region. The source of the chloramphenicol resistance gene, pCGN565 is a cloning vector based on pUC12-cm (K. Buckley Ph.D. Thesis, Regulation and expression of the phi X174 lysis gene, University of California, San Diego, 1985), but containing pUC18 linkers (Yanisch-Perron, *et al.*, *Gene* (1985) *53*:103-119). pCGN565 is digested with *Hha*I and the fragment containing the chloramphenicol resistance gene is excised, blunted by use of mung bean nuclease, and inserted into the *Eco*RV site of Bluescript KS- (Stratagene: La Jolla, CA) to create pCGN2008. The chloramphenicol resistance gene of pCGN2008 is removed by *Eco*RI/*Hin*dIII digestion. After treatment with Klenow enzyme to blunt the ends, the fragment is ligated to *Dra*I digested Bluescript KS+. A clone that has the *Dra*I fragment containing ampicillin resistance replaced with the chloramphenicol resistance is chosen and named pCGN2015. The linker region of pCGN2015 is modified to provide pCGN3686, which contains the following restriction digestion sites, 5' to 3' in the lacZ linker region: *Pst*I, *Bgl*II, *Xho*I, *Hin*cII, *Sal*I, *Hin*dIII, *Eco*RV, *Eco*RI, *Pst*I, *Sma*I, *Bam*HI, *Spe*I, *Xba*I and *Sac*I.

An *Xho*I linker is inserted at the *Xba*I site of pCGN7582. The *Bgl*II-*Xho*I fragment containing the reductase gene is isolated and cloned into *Bgl*II-*Xho*I digested pCGN3223. The

resulting plasmid, which lacks the 5' untranslated leader sequence from the jojoba gene, is designated pCGN7802. The napin/reductase fragment from pCGN7802 is excised with *Hin*dIII and cloned into *Hin*dIII digested pCGN1578 to yield pCGN7589.

pCGN7586 and pCGN7589 are transformed into *Agrobacterium* cells, such as of strain EHA101 (Hood *et al.*, *J. Bacteriol* (1986) *168*:1291-1301), by the method of Holsters *et al.* (*Mol. Gen. Genet.* (1978) *163*:181-187) and used in plant transformation methods as described below.

Example 9 - Plant Transformation Methods

A variety of methods have been developed to insert a DNA sequence of interest into the genome of a plant host to obtain the transcription or transcription and translation of the sequence to effect phenotypic changes.

Brassica Transformation

Seeds of high erucic acid, such as cultivar Reston, or Canola-type varieties of *Brassica napus* are soaked in 95% ethanol for 2 min. surface sterilized in a 1.0% solution of sodium hypochlorite containing a drop of Tween 20 for 45 min., and rinsed three times in sterile, distilled water. Seeds are then plated in Magenta boxes with 1/10th concentration of Murashige minimal organics medium (Gibco; Grand Island,NY) supplemented with pyriodoxine (50µg/l), nicotinic acid (50µg/l), glycine (200µg/l), and 0.6% Phytagar (Gibco) pH 5.8. Seeds are germinated in a Percival chamber at 22°C. in a 16 h photoperiod with cool fluorescent and red light of intensity approximately 65µ Einsteins per square meter per second ($\mu Em^{-2}s^{-1}$).

Hypocotyls are excised from 5-7 day old seedlings, cut into pieces approximately 4mm in length, and plated on feeder plates (Horsch *et al.*, *Science* (1985) *227*:1229-1231). Feeder plates are prepared one day before use by plating 1.0ml of a tobacco suspension culture onto a petri plate (100x25mm) containing about 30ml MS salt base (Carolina Biological, Burlington, NC) 100mg/l inositol,

1.3mg/l thiamine-HCl, 200mg KH_2PO_4 with 3% sucrose, 2,4-D (1.0mg/l), 0.6% w/v Phytagar, and pH adjusted to 5.8 prior to autoclaving (MS 0/1/0 medium). A sterile filter paper disc (Whatman 3mm) is placed on top of the feeder layer prior to use. Tobacco suspension cultures are subcultured weekly by transfer of 10ml of culture into 100ml fresh MS medium as described for the feeder plates with 2,4-D (0.2mg/l), Kinetin (0.1mg/l). In experiments where feeder cells are not used hypocotyl explants are cut and placed onto a filter paper disc on top of MS0/1/0 medium. All hypocotyl explants are preincubated on feeder plates for 24 h. at 22°C in continuous light of intensity 30$\mu Em^{-2}s^{-1}$ to 65$\mu EM^{-2}S^{-1}$.

Single colonies of *A. tumefaciens* strain EHA101 containing a binary plasmid with the desired gene construct are transferred to 5ml MG/L broth and grown overnight at 30°C. Hypocotyl explants are immersed in 7-12ml MG/L broth with bacteria diluted to $1x10^8$ bacteria/ml and after 10-25 min. are placed onto feeder plates. Per liter MG/L broth contains 5g mannitol, 1g L-Glutamic acid or 1.15g sodium glutamate, 0.25g kH_2PO_4, 0.10g NaCl, 0.10g $MGSO_4 \cdot 7H_20$, 1mg biotin, 5g tryptone, and 2.5g yeast extract, and the broth is adjusted to pH 7.0. After 48 hours of co-incubation with *Agrobacterium*, the hypocotyl explants are transferred to B5 0/1/0 callus induction medium which contains filter sterilized carbenicillin (500mg/l, added after autoclaving) and kanamycin sulfate (Boehringer Mannheim; Indianapolis, IN) at concentrations of 25mg/l.

After 3-7 days in culture at 65$\mu EM^{-2}S^{-1}$ continuous light, callus tissue is visible on the cut surface and the hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented with 3mg/l benzylaminopurine, 1mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contains carbenicillin (500mg/l) and kanamycin sulfate (25mg/l). Hypocotyl explants are subcultured onto fresh shoot induction medium every two weeks.

Shoots regenerate from the hypocotyl calli after one to three months. Green shoots at least 1cm tall are excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, carbenicillin (300mg/l), kanamycin sulfate (50mg/l) and 0.6% w/v Phytagar). After 2-4 weeks shoots which remain green are cut at the base and transferred to Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2mg/l indolebutyric acid, 50mg/l kanamycin sulfate and 0.6% Phytagar). Green rooted shoots are tested for thioesterase activity.

Arabidposis Transformation

Transgenic *Arabidopsis thaliana* plants may be obtained by *Agrobacterium*-mediated transformation as described by Valverkens *et al.*, (*Proc. Nat. Acad. Sci.* (1988) *85*:5536-5540). Constructs are transformed into *Agrobacterium* cells, such as of strain EHA101 (Hood *et al.*, *J. Bacteriol* (1986) *168*:1291-1301), by the method of Holsters *et al.* (*Mol. Gen. Genet.* (1978) *163*:181-187).

Peanut Transformation

DNA sequences of interest may be introduced as expression cassettes, comprising at least a promoter region, a gene of interest, and a termination region, into a plant genome via particle bombardment.

Briefly, tungsten or gold particles of a size ranging from 0.5mM-3mM are coated with DNA of an expression cassette. This DNA may be in the form of an aqueous mixture or a dry DNA/particle precipitate.

Tissue used as the target for bombardment may be from cotyledonary explants, shoot meristems, immature leaflets, or anthers. The bombardment of the tissue with the DNA-coated particles is carried out using a Biolistics particle gun (Dupont; Wilmington, DE). The particles are placed in the barrel at variable distances ranging from 1cm-14cm from the barrel mouth. The tissue to be bombarded is placed beneath the stopping plate; testing is performed

on the tissue at distances up to 20cm. At the moment of discharge, the tissue is protected by a nylon net or a combination of nylon nets with mesh ranging from 10mM to 300mM.

Following bombardment, plants may be regenerated following the method of Atreya, *et al.*, (*Plant Science Letters* (1984) *34*:379-383). Briefly, embryo axis tissue or cotyledon segments are placed on MS medium (Murashige and Skoog, *Physio. Plant.* (1962) *15*:473) (MS plus 2.0 mg/l 6-benzyladenine (BA) for the cotyledon segments) and incubated in the dark for 1 week at 25 ± 2°C and are subsequently transferred to continuous cool white fluorescent light (6.8 W/m^2). On the 10th day of culture, the plantlets are transferred to pots containing sterile soil, are kept in the shade for 3-5 days are and finally moved to greenhouse. The putative transgenic shoots are rooted. Integration of exogenous DNA into the plant genome may be confirmed by various methods know to those skilled in the art.

Example 10 - Analysis of Transformed Plants for Wax Production

Seeds or other plant material from transformed plants may be analyzed for wax synthase activity using the wax synthase assay methods described in Example 1.

Plants which have both the reductase and wax synthase constructs are also assayed to measure wax production. Such plants may be prepared by *Agrobacterium* transformation methods as described above. Plants having both of the desired gene constructs may be prepared by co-transformation with reductase and wax synthase constructs or by combining the wax synthase and reductase constructs on a single plant transformation binary vector. In addition, re-transformation of either wax synthase expressing plants or reductase expressing plants with constructs encoding the other desired gene sequence may also be used to provide such reductase and wax synthase expressing plants. Alternatively, transgenic plants expressing reductase produced by methods described herein

may be crossed with plants expressing wax synthase which have been similarly produced. In this manner, known methods of plant breeding are used to provide reductase and wax synthase expressing transgenic plants.

Such plants may be assayed for the presence of wax esters, for example by separation of TAG from wax esters as described by Tani *et al.* (*supra*). GC analysis methods may be used to further analyze the resulting waxes, for example as described by Pina et al. (Lipids (1987) 22(5):358-361.

The above results demonstrate the ability to obtain partially purified wax synthase proteins which are active in the formation of wax esters from fatty alcohol and fatty acyl substrates. Methods to obtain the wax synthase proteins and amino acid sequences thereof are provided. In addition wax synthase nucleic acid sequences obtained from the amino acid sequences are also provided. These nucleic acid sequences may be manipulated to provide for transcription of the sequences and/or expression of wax synthase proteins in host cells, which proteins may be used for a variety of applications. Such applications include the production of wax ester compounds when the wax synthase is used in host cells having a source of fatty alcohol substrates, which substrates may be native to the host cells or supplied by use of recombinant constructs encoding a fatty acyl reductase protein which is active in the formation of alcohols from fatty acyl substrates.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be readily apparent to those of ordinary skill in the art in light of the teaching of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A recombinant DNA construct comprising a nucleic acid sequence which encodes at least a portion of a wax synthase protein, and a heterologous DNA sequence not naturally associated with said wax synthase encoding sequence.

2. The construct of Claim 1 wherein said wax synthase is active toward a fatty acyl-CoA substrate.

3. The construct of Claim 1 wherein said wax synthase is active toward a fatty acyl substrate having a carbon chain of the formula C_{2x} wherein X is selected from the group 6-12.

4. The construct of Claim 1 wherein said wax synthase is active toward a fatty alcohol substrate having a carbon chain of the formula C_{2x} wherein X is selected from the group 6-12.

5. The construct of Claim 1 wherein said wax synthase encoding sequence is from a seed plant.

6. The construct of Claim 1 wherein said wax synthase encoding sequence is from jojoba.

7. The construct of Claim 1 further comprising a promoter which provides for at least transcription of said wax synthase encoding sequence in a host cell.

8. The construct of Claim 7 wherein said promoter provides for expression of said wax synthase encoding sequence in a plant cell.

9. The construct of Claim 8 wherein said plant cell is a plant embryo seed cell.

10. The construct of of Claim 7 wherein said promoter provides for expression of said wax synthase encoding sequence in a bacterial cell.

11. The construct of Claim 8 wherein said promoter is from a gene preferentially expressed in a plant seed embryo cell.

12. A cell comprising a construct according to Claim 1.

13. A plant cell comprising a construct according to Claim 1.

14. A host cell comprising a recombinant construct comprising a nucleic acid sequence which encodes a fatty acyl reductase protein; and

a recombinant construct comprising a nucleic acid sequence which encodes a wax synthase protein, wherein said wax synthase encoding sequence is heterologous to said host cell, and wherein said reductase and wax synthase encoding sequences are under the regulatory control of promoters functional in said host cell.

15. The host cell of Claim 14, wherein said host cell is a plant cell.

16. The plant cell of Claim 15, wherein said plant cell is a *Brassica* plant cell.

17. A *Brassica* plant cell comprising a construct according to Claim 1.

18. A procaryotic cell comprising a seed-plant wax synthase.

19. A method of producing a wax synthase in a host cell comprising the steps of

growing a host cell comprising a recombinant construct,

said construct comprising a nucleic acid sequence which encodes a wax synthase, wherein said wax synthase encoding sequence is under the control of regulatory elements functional in said cell,

under conditions which will cause the expression of said wax synthase encoding sequence.

20. The method of Claim 19 wherein said host cell is a procaryote.

21. The method of Claim 19 wherein said host cell is a seed plant embryo cell.

22. The method of Claim 21 wherein said seed plant is *Brassica*.

23. The method of Claim 19 wherein said wax synthase encoding sequence is from a seed plant.

24. A method of producing a wax ester in a host cell comprising the steps of

growing a host cell comprising a recombinant construct,

said construct comprising a nucleic acid sequence which encodes a wax synthase, wherein said wax synthase encoding sequence is under the control of regulatory elements functional in said host cell, and wherein said host cell comprises a fatty alcohol substrate of said wax synthase,

under conditions which will cause the expression of said wax synthase sequence.

25. The method of Claim 24, wherein said fatty alcohol substrate of said wax synthase is produced in said host cell as the result of expression of a fatty acyl reductase encoding sequence from a heterologous recombinant construct.

26. The method of Claim 24 or 25, wherein said host cell is a plant cell.

27. The method of Claim 26, wherein said plant cell is a seed plant embryo cell.

28. The method of Claim 27, wherein said plant is *Brassica*.

29. A host cell comprising a wax ester produced according to the method of Claim 24 or 25.

30. A host cell of Claim 29, wherein said host cell is a plant cell.

31. A host cell of Claim 30, wherein said plant cell is a *Brassica* seed embryo cell.

32. A *Brassica* seed cell, wherein the internal lipid reserves of said seed cell comprise wax esters.

33. A *Brassica* seed cell of Claim 32, wherein said wax esters are long chain wax esters.

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AAATCCTCCA CTCATACACT CCACTTCTCT CTCTCTCTCT CTCTCTCTGA AACAATTTGA    60

GTAGCAAACT TAAAAGAAA ATG GAG GAA ATG GGA AGC ATT TTA GAG TTT CTT    112
                     Met Glu Glu Met Gly Ser Ile Leu Glu Phe Leu
                     1               5                  10

GAT AAC AAA GCC ATT TTG GTC ACT GGT GCT ACT GGC TCC TTA GCA AAA    160
Asp Asn Lys Ala Ile Leu Val Thr Gly Ala Thr Gly Ser Leu Ala Lys
            15                  20                  25

ATT TTT GTG GAG AAG GTA CTG AGG AGT CAA CCG AAT GTG AAG AAA CTC    208
Ile Phe Val Glu Lys Val Leu Arg Ser Gln Pro Asn Val Lys Lys Leu
        30                  35                  40

TAT CTT CTT TTG AGA GCA ACC GAT GAC GAG ACA GCT GCT CTA CGC TTG    256
Tyr Leu Leu Leu Arg Ala Thr Asp Asp Glu Thr Ala Ala Leu Arg Leu
    45                  50                  55

CAA AAT GAG GTT TTT GGA AAA GAG TTG TTC AAA GTT CTG AAA CAA AAT    304
Gln Asn Glu Val Phe Gly Lys Glu Leu Phe Lys Val Leu Lys Gln Asn
60                  65                  70                  75
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FIG. 1A

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TTA GGT GCA AAT TTC TAT TCC TTT GTA TCA GAA AAA GTG ACT GTA GTA     352
Leu Gly Ala Asn Phe Tyr Ser Phe Val Ser Glu Lys Val Thr Val Val
                80                  85                  90

CCC GGT GAT ATT ACT GGT GAA GAC TTG TGT CTC AAA GAC GTC AAT TTG     400
Pro Gly Asp Ile Thr Gly Glu Asp Leu Cys Leu Lys Asp Val Asn Leu
            95                  100                 105

AAG GAA GAA ATG TGG AGG GAA ATC GAT GTT GTT GTC AAT CTA GCT GCT     448
Lys Glu Glu Met Trp Arg Glu Ile Asp Val Val Val Asn Leu Ala Ala
        110                 115                 120

ACA ATC AAC TTC ATT GAA AGG TAC GAC GTG TCT CTG CTT ATC AAC ACA     496
Thr Ile Asn Phe Ile Glu Arg Tyr Asp Val Ser Leu Leu Ile Asn Thr
    125                 130                 135

TAT GGA GCC AAG TAT GTT TTG GAC TTC GCG AAG AAG TGC AAC AAA TTA     544
Tyr Gly Ala Lys Tyr Val Leu Asp Phe Ala Lys Lys Cys Asn Lys Leu
140                 145                 150                 155

AAG ATA TTT GTT CAT GTA TCT ACT GCT TAT GTA TCT GGA GAG AAA AAT     592
Lys Ile Phe Val His Val Ser Thr Ala Tyr Val Ser Gly Glu Lys Asn
                160                 165                 170
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FIG. 1B

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GGG TTA ATA CTG GAG AAG CCT TAT TAT ATG GGC GAG TCA CTT AAT GGA      640
Gly Leu Ile Leu Glu Lys Pro Tyr Tyr Met Gly Glu Ser Leu Asn Gly
            175                 180                 185

AGA TTA GGT CTG GAC ATT AAT GTA GAG AAG AAA CTT GTG GAG GCA AAA      688
Arg Leu Gly Leu Asp Ile Asn Val Glu Lys Lys Leu Val Glu Ala Lys
        190                 195                 200

ATC AAT GAA CTT CAA GCA GCG GGG GCA ACG GAA AAG TCC ATT AAA TCG      736
Ile Asn Glu Leu Gln Ala Ala Gly Ala Thr Glu Lys Ser Ile Lys Ser
    205                 210                 215

ACA ATG AAG GAC ATG GGC ATC GAG AGG GCA AGA CAC TGG GGA TGG CCA      784
Thr Met Lys Asp Met Gly Ile Glu Arg Ala Arg His Trp Gly Trp Pro
220                 225                 230                 235

AAT GTG TAT GTA TTC ACC AAG GCA TTA GGG GAG ATG CTT TTG ATG CAA      832
Asn Val Tyr Val Phe Thr Lys Ala Leu Gly Glu Met Leu Leu Met Gln
                240                 245                 250

TAC AAA GGG GAC ATT CCG CTT ACT ATT ATT CGT CCC ACC ATC ATC ACC      880
Tyr Lys Gly Asp Ile Pro Leu Thr Ile Ile Arg Pro Thr Ile Ile Thr
            255                 260                 265
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FIG. 1C

```
AGC ACT TTT AAA GAG CCC TTT CCT GGT TGG GTT GAA GGT GTC AGG ACC      928
Ser Thr Phe Lys Glu Pro Phe Pro Gly Trp Val Glu Gly Val Arg Thr
        270                 275                 280

ATC GAT AAT GTA CCT GTA TAT TAT GGT AAA GGG AGA TTG AGG TGT ATG      976
Ile Asp Asn Val Pro Val Tyr Tyr Gly Lys Gly Arg Leu Arg Cys Met
    285                 290                 295

CTT TGC GGA CCC AGC ACA ATA ATT GAC CTG ATA CCG GCA GAT ATG GTC     1024
Leu Cys Gly Pro Ser Thr Ile Ile Asp Leu Ile Pro Ala Asp Met Val
300                 305                 310                 315

GTG AAT GCA ACG ATA GTA GCC ATG GTG GCG CAC GCA AAC CAA AGA TAC     1072
Val Asn Ala Thr Ile Val Ala Met Val Ala His Ala Asn Gln Arg Tyr
                320                 325                 330

GTA GAG CCG GTG ACA TAC CAT GTG GGA TCT TCA GCG GCG AAT CCA ATG     1120
Val Glu Pro Val Thr Tyr His Val Gly Ser Ser Ala Ala Asn Pro Met
            335                 340                 345

AAA CTG AGT GCA TTA CCA GAG ATG GCA CAC CGT TAC TTC ACC AAG AAT     1168
Lys Leu Ser Ala Leu Pro Glu Met Ala His Arg Tyr Phe Thr Lys Asn
        350                 355                 360
```

FIG. 1D

```
CCA TGG ATC AAC CCG GAT CGC AAC CCA GTA CAT GTG GGT CGG GCT ATG    1216
Pro Trp Ile Asn Pro Asp Arg Asn Pro Val His Val Gly Arg Ala Met
    365                 370                 375

GTC TTC TCC TCC TTC TCC ACC TTC CAC CTT TAT CTC ACC CTT AAT TTC    1264
Val Phe Ser Ser Phe Ser Thr Phe His Leu Tyr Leu Thr Leu Asn Phe
380                 385                 390                 395

CTC CTT CCT TTG AAG GTA CTG GAG ATA GCA AAT ACA ATA TTC TGC CAA    1312
Leu Leu Pro Leu Lys Val Leu Glu Ile Ala Asn Thr Ile Phe Cys Gln
                400                 405                 410

TGG TTC AAG GGT AAG TAC ATG GAT CTT AAA AGG AAG ACG AGG TTG TTG    1360
Trp Phe Lys Gly Lys Tyr Met Asp Leu Lys Arg Lys Thr Arg Leu Leu
            415                 420                 425

TTG CGT TTA GTA GAC ATT TAT AAA CCC TAC CTC TTC TTC CAA GGC ATC    1408
Leu Arg Leu Val Asp Ile Tyr Lys Pro Tyr Leu Phe Phe Gln Gly Ile
        430                 435                 440

TTT GAT GAC ATG AAC ACT GAG AAG TTG CGG ATT GCT GCA AAA GAA AGC    1456
Phe Asp Asp Met Asn Thr Glu Lys Leu Arg Ile Ala Ala Lys Glu Ser
    445                 450                 455
```

FIG. 1E

```
ATA GTT GAA GCT GAT ATG TTT TAC TTT GAT CCC AGG GCA ATT AAC TGG   1504
Ile Val Glu Ala Asp Met Phe Tyr Phe Asp Pro Arg Ala Ile Asn Trp
460                 465                 470                 475

GAA GAT TAC TTC TTG AAA ACT CAT TTC CCA GGN GTC GTA GAG CAC GTT   1552
Glu Asp Tyr Phe Leu Lys Thr His Phe Pro Gly Val Val Glu His Val
                480                 485                 490

CTT AAC TAAAAGTTAC GGTACGAAAA TGAGAAGATT GGAATGCATG CACCGAAAGN    1608
Leu Asn

NCAACATAAA AGACGTGGTT AAAGTCATGG TCAAAAAAGA AATAAAATGC AGTTAGGTTT 1668

GTGTTGCAGT TTTGATTCCT TGTATTGTTA CTTGTACTTT TGATCTTTTT CTTTTTTAAT 1728

GAAATTTCTC TCTTTGTTTT GTGAAAAAAA AAAAAAAAAA GAGCTCCTGC AGAAGCTT   1786
```

FIG. 1F

```
GGAACTCCAT CCCTTCCTCC CTCACTCCTC TCTCTACA ATG AAG GCC AAA ACA ATC   56
                                          Met Lys Ala Lys Thr Ile
                                           1               5

ACA AAC CCG GAG ATC CAA GTC TCC ACG ACC ATG ACC ACC ACG ACC ACG    104
Thr Asn Pro Glu Ile Gln Val Ser Thr Thr Met Thr Thr Thr Thr Thr
           10                  15                  20

ACT ATG ACC GCC ACT CTC CCC AAC TTC AAG TCC TCC ATC AAC TTA CAC    152
Thr Met Thr Ala Thr Leu Pro Asn Phe Lys Ser Ser Ile Asn Leu His
       25                  30                  35

CAC GTC AAG CTC GGC TAC CAC TAC TTA ATC TCC AAT GCC CTC TTC CTC    200
His Val Lys Leu Gly Tyr His Tyr Leu Ile Ser Asn Ala Leu Phe Leu
   40                  45                  50

GTA TTC ATC CCC CTT TTG GGC CTC GCT TCG GCC CAT CTC TCC TCC TTC    248
Val Phe Ile Pro Leu Leu Gly Leu Ala Ser Ala His Leu Ser Ser Phe
55                 60                  65                  70
```

FIG. 2A

```
TCG GCC CAT GAC TTG TCC CTG CTC TTC GAC CTC CTT CGC CGC AAC CTC     296
Ser Ala His Asp Leu Ser Leu Leu Phe Asp Leu Leu Arg Arg Asn Leu
                 75                  80                  85

CTC CCT GTT GTC GTT TGT TCT TTC CTC TTC GTT TTA TTA GCA ACC CTA     344
Leu Pro Val Val Val Cys Ser Phe Leu Phe Val Leu Leu Ala Thr Leu
            90                  95                 100

CAT TTC TTG ACC CGG CCC AGG AAT GTC TAC TTG GTG GAC TTT GGA TGC     392
His Phe Leu Thr Arg Pro Arg Asn Val Tyr Leu Val Asp Phe Gly Cys
        105                 110                 115

TAT AAG CCT CAA CCG AAC CTG ATG ACA TCC CAC GAG ATG TTC ATG GAC     440
Tyr Lys Pro Gln Pro Asn Leu Met Thr Ser His Glu Met Phe Met Asp
    120                 125                 130

CGG ACC TCC CGG GCC GGG TCG TTT TCT AAG GAG AAT ATT GAG TTT CAG     488
Arg Thr Ser Arg Ala Gly Ser Phe Ser Lys Glu Asn Ile Glu Phe Gln
135                 140                 145                 150

AGG AAG ATC TTG GAG AGG GCC GGT ATG GGT CGG GAA ACC TAT GTC CCC     536
Arg Lys Ile Leu Glu Arg Ala Gly Met Gly Arg Glu Thr Tyr Val Pro
                155                 160                 165
```

FIG. 2B

```
GAA TCC GTC ACT AAG GTG CCC GCC GAG CCG AGC ATA GCA GCA GCC AGG      584
Glu Ser Val Thr Lys Val Pro Ala Glu Pro Ser Ile Ala Ala Ala Arg
            170                 175                 180

GCC GAG GCG GAG GAG GTG ATG TAC GGG GCG ATC GAC GAG GTG TTG GAG      632
Ala Glu Ala Glu Glu Val Met Tyr Gly Ala Ile Asp Glu Val Leu Glu
        185                 190                 195

AAG ACG GGG GTG AAG CCG AAG CAG ATA GGA ATA CTG GTG GTG ANC TGC      680
Lys Thr Gly Val Lys Pro Lys Gln Ile Gly Ile Leu Val Val Xxx Cys
    200                 205                 210

AGC TTG TTT AAC CCA ACG CCG TCG CTG TCA TCC ATG ATA GTT AAC CAT      728
Ser Leu Phe Asn Pro Thr Pro Ser Leu Ser Ser Met Ile Val Asn His
215                 220                 225                 230

TAC AAG CTN AGG GGT AAT ATA CTT AGC TAT AAT CTT GGT GGC ATG GGT      776
Tyr Lys Leu Arg Gly Asn Ile Leu Ser Tyr Asn Leu Gly Gly Met Gly
                235                 240                 245

TGC AGT GCT GGG CTC ATT TCC ATT GAT CTT GCC AAG GAC CTC CTA CAG      824
Cys Ser Ala Gly Leu Ile Ser Ile Asp Leu Ala Lys Asp Leu Leu Gln
            250                 255                 260
```

FIG. 2C

```
GTT TAC CGT AAA AAC ACA TAT GTG TTA GTA GTG AGC ACG GAA AAC ATG    872
Val Tyr Arg Lys Asn Thr Tyr Val Leu Val Val Ser Thr Glu Asn Met
        265                 270                 275

ACC CTT AAT TGG TAC TGG GGC AAT GAC CGC TCC ATG CTT ATC ACC AAC    920
Thr Leu Asn Trp Tyr Trp Gly Asn Asp Arg Ser Met Leu Ile Thr Asn
    280                 285                 290

TGC CTA TTT CGC ATG GGT GGC GCT GCC ATC ATC CTC TCA AAC CGC TGG    968
Cys Leu Phe Arg Met Gly Gly Ala Ala Ile Ile Leu Ser Asn Arg Trp
295                 300                 305                 310

CGT GAT CGT CGC CGA TCC AAG TAC CAA CTC CTT CAT ACA GTA CGC ACC   1016
Arg Asp Arg Arg Arg Ser Lys Tyr Gln Leu Leu His Thr Val Arg Thr
                315                 320                 325

CAC AAG GGC GCT GAC GAC AAG TCC TAT AGA TGC GTC TTA CAA CAA GAA   1064
His Lys Gly Ala Asp Asp Lys Ser Tyr Arg Cys Val Leu Gln Gln Glu
            330                 335                 340

GAT GAA AAT AAC AAG GTA GGT GTT GCC TTA TCC AAG GAT CTG ATG GCA   1112
Asp Glu Asn Asn Lys Val Gly Val Ala Leu Ser Lys Asp Leu Met Ala
        345                 350                 355
```

FIG. 2D

```
GTT GCC GGT GAA GCC CTA AAG GCC AAC ATC ACG ACC CTT GGT CCC CTC   1160
Val Ala Gly Glu Ala Leu Lys Ala Asn Ile Thr Thr Leu Gly Pro Leu
    360                 365                 370

GTG CTC CCC ATG TCA GAA CAA CTC CTC TTC TTT GCC ACC TTA GTG GCA   1208
Val Leu Pro Met Ser Glu Gln Leu Leu Phe Phe Ala Thr Leu Val Ala
375                 380                 385                 390

CGT AAG GTC TTC AAG ATG ACG AAC GTG AAG CCA TAC ATC CCA GAT TTC   1256
Arg Lys Val Phe Lys Met Thr Asn Val Lys Pro Tyr Ile Pro Asp Phe
                395                 400                 405

AAG TTG GCA GCG AAC GAC TTC TGC ATC CAT GCA GGA GGC AAA GCA GTG   1304
Lys Leu Ala Ala Asn Asp Phe Cys Ile His Ala Gly Gly Lys Ala Val
            410                 415                 420

TTG GAT GAG CTC GAG AAG AAC TTG GAG TTG ACG CCA TGG CAC CTT GAA   1352
Leu Asp Glu Leu Glu Lys Asn Leu Glu Leu Thr Pro Trp His Leu Glu
        425                 430                 435

CCC TCG AGG ATG ACA CTG TAT AGG TTT GGG AAC ACA TCG AGT AGC TCA   1400
Pro Ser Arg Met Thr Leu Tyr Arg Phe Gly Asn Thr Ser Ser Ser Ser
    440                 445                 450
```

FIG. 2E

```
TTA TGG TAC GAG TTG GCA TAC GCT GAA GCA AAA GGG AGG ATC CGT AAG   1448
Leu Trp Tyr Glu Leu Ala Tyr Ala Glu Ala Lys Gly Arg Ile Arg Lys
455                 460                 465                 470

GGT GAT CGA ACT TGG ATG ATT GGA TTT GGT TCA GGT TTC AAG TGT AAC   1496
Gly Asp Arg Thr Trp Met Ile Gly Phe Gly Ser Gly Phe Lys Cys Asn
                475                 480                 485

AGT GTT GTG TGG AGG GCT TTG AGG AGT GTC AAT CCG GCT AGA GAG AAG   1544
Ser Val Val Trp Arg Ala Leu Arg Ser Val Asn Pro Ala Arg Glu Lys
            490                 495                 500

AAT CCT TGG ATG GAT GAA ATT GAG AAG TTC CCT GTC CAT GTG CCT AAA   1592
Asn Pro Trp Met Asp Glu Ile Glu Lys Phe Pro Val His Val Pro Lys
        505                 510                 515

ATC GCA CCT ATC GCT TCG TAGAACTGCT AGGATGTGAT TAGTAATGAA          1640
Ile Ala Pro Ile Ala Ser
    520

AAATGTGTAT TATGTTAGTG ATGTAGAAAA AGAAACTTTA GTTGATGGGT GAGAACATGT 1700

CTCATTGAGA ATAACGTGTG CATCGTTGTG TTG                              1733
```

FIG. 2F

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/09863

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/54; C12N9/10; C12P7/64; C12N15/82
C12N15/05; //C12N9/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
O,A	ABSTRACT FROM THE SOUTHWEST CONSORTIUM ON PLANT GENETICS AND WATER RESOURCES, 5TH ANNUAL MEETING April 1990, LAS CRUCES NM GUNTER F. WILDNER ET AL. 'Wax ester biosynthesis in euglena gracilis' cited in the application see the whole document ---	
A	LIPIDS vol. 14, no. 7, 1979, pages 651 - 662 MICHAEL R. POLLARD ET AL. 'Studies on biosynthesis of waxes by developing Jojoba seed. II.The demonstration of wax biosynthesis by cell-free homogenates' cited in the application see abstract see page 651, left column, paragraph 2 - right column, paragraph 1 --- -/--	

° Special categories of cited documents :[10]

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 FEBRUARY 1993	26. 02. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF LIPID RESEARCH vol. 27, no. 4, April 1986, pages 404 - 411 P.E. KOLATTUKUDY ET AL. 'Acyl-CoA reductase and acyl-CoA: fatty alcohol acyl tranferase in the microsomal preparation from the bovine meibomian gland' see abstract -----	